REPORT

OF THE

NODA INSTITUTE

FOR

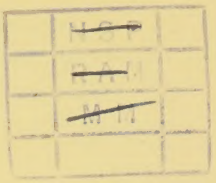
SCIENTIFIC RESEARCH

No. 4 1960

CONTENTS

Hiroshi ŌNISHI: Studies on Osmophilic Yeasts Part VII, VIII, IX, X 1
Shin-ichi SUGIYAMA, Keiji YANO, Kazuo KOMAGATA, Masaji KAZUMA and Kei ARIMA: Metabolism of Aromatic Compounds by Microbes Part VI 32
Shin-ichi SUGIYAMA, Keiji YANO, Kazuo KOMAGATA and Kei ARIMA: Metabolism of Aromatic Compounds by Microbes Part VII 38
Shin-ichi SUGIYAMA, Keiji YANO and Kei ARIMA: Metabolism of Aromatic Compounds by Microbes Part VIII 44
Shin-ichi SUGIYAMA, Kirosato TANAKA, Keiji YANO and Kei ARIMA: Metabolism of Aromatic Compounds by Microbes Part IX 50
Kenji SAKAGUCHI: Betaine as a Growth Factor for *Pediococcus soyae* Part VIII . . 57
Zenzirō SUGISAKI: Studies on L-Valine Fermentation Part I, II. 65

Published by
Noda Institute for Scientific Research
Noda-shi, Chiba-ken, Japan



Digitized by the Internet Archive
in 2025

https://archive.org/details/report-of-the-noda-institute-for-scientific-research_1960_4

Studies on Osmophilic Yeasts*

Part VII. Production of Polyalcohols by *Saccharomyces rouxii* in the Concentrated Media of Sodium Chloride and Sugars, and Identification of Polyalcohols Produced

By Hiroshi Ōnishi

Identification of polyalcohols which were produced in large amounts by *Saccharomyces rouxii* under aerobic fermentation in the concentrated media of sodium chloride and sugar, was carried out by isolating these products in crystalline form from the fermented broth. The products were determined as glycerol and D-arabitol. In the concentrated medium of sodium chloride, about 70% of the polyalcohols produced was glycerol, while in the concentrated sugar medium, the formation of D-arabitol was more markable than glycerol.

INTRODUCTION

In the previous paper[1], the author reported that *Saccharomyces rouxii*, which is a typical salt-tolerant yeast and plays an important role in soy-brewing, is found to ferment glucose giving high yields of glycerol in the high saline medium under aerobic conditions, though it produces a small amount of glycerol in the ordinary medium. From the results of paper chromatography and partition chromatography using celite, it was also reported that the majority of values obtained as crude glycerol is found to be true glycerol, a small amount of polyalcohol other than glycerol being detected.

Spencer and Sallans[2] found that osmophilic *Zygosaccharomyces* isolated from fermenting honey and other sources produces one or more of the following sugar alcohols, glycerol, erythritol, D-arabitol, mannitol and two unidentified polyhydric alcohols under aerobic conditions, and carried out detailed studies on the factors favoring glycerol production[3]. Peterson et al.[4] also investigated foctors affecting production of glycerol and D-arabitol employing representative yeasts of the genus *Zygosaccharomyces*.

It is noted that all of these studies are carried out in the ordinary medium and not in the concentrated saline medium. In the case of *Sacch. rouxii*, a salt-tolerant soy yeast, effect of NaCl concentrations of the medium on the yields of glycerol was very remarkable as previously stated[1]. For the purpose of identification of polyacohols produced by aerobic fermentation in the two concentrated media of glucose and sodium chloride, isolation in crystalline form from the fermented broths was carried out, from which they were determined as glycerol ane D-arabitol. In the concentrated salt medium, about 70% of polyalkohols produced were glycerol, while in the concentrated glucose medium, a large amount of D-arabitol was produced than in case of glycerol.

EXPERIMENTAL AND RESULTS

[I] Cultural condition.

Yeast strain employed: *Sacch. rouxii* m 3[1]

The two media used were as follows: (1) the con-

*Reprinted by permission of Agr. Chem. Soc. Japan from Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 2.

1) H. Ōnishi, This Bulletih, **23**, 359 (1959).

2) J. F. T. Spencer and H. R. Sallans. *Can. J. Microbiol.*, **2**, 72 (1956).

3) J. F. T. Spencer. J. M. Roxburgh and H. R. Sallans, *J. Agr. Food Chem.*, **5**, 64 (1957).

4) W. H. Peterson, W. F. Hendershot and G. I. Hajny, *App Microbiol.*, **6**, 349 (1958).

centrated saline medium: glucose 10%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCl_2 \cdot 2H_2O$ 0.01%, NaCl 18.0%, casamino acid 0.4%, yeast-ext. 0.1%, pH 4.4, (2) the concentrated glucose medium: glucose 30%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCl_2 \cdot 2H_2O$ 0.01%, NaCl 0.01%, casamino acid 0.4%, yeast-ext. 0.1%, pH 4.8.

Eighty ml of these media were placed in a 500 ml shake-flask, sterilized and then one drop of fresh culture suspension was inoculated. The flasks were shaken on a reciprocal shaker operating at 120 r.p.m. with a stroke of 75 mm at 30°. After 9 days' incubation, the fermentation products were subjected to analysis.

[II] Analysis of the fermented broth.

The cleared fermentation solutions from which the yeast cells were removed by filtration were analysed according to the following methods.

Glucose: The Fehling-Lehmann-Schoorl method[5].

Ethanol: After distillation in neutral (pH 7.6~7.8), the oxidation method by dichromate was applied.

pH: pH test paper (Tokyo Roshi Kaisha)

Total acid: Ether extracts in acid reaction with H_2SO_4, are titrated with N/20 NaOH.

Total polyalcohols (calculated as glycerol): the method of Lambert and Neish[6] as described by Neish[7].

Total polyalcohols were determined by oxidation with periodic acid followed by colorimetric determination of formaldehyde formed with a chromotropic acid reagent. The values obtained were corrected for glucose presented by running a standard glucose sample in parallel with the fermentation solutions.

True glycerol: Glycerol was separated on water-celite columns using a mixture of 25% benzene and 75% *n*-butanol saturated with water as a developing solvent and analysed by the above periodic oxidation procedure. The results are shown in Table I.

[III] Isolation of each polyalcohol from the fermented broths.

As shown in Table I, 75% of total polyalcohols (calculated as glycerol) produced in the concentrated saline medium were ture glycerol, while in the concentrated glucose medium, polyalcohol other than glycerol was formed in larger quantities than glycerol: that is to say, the ratio of glycerol to other polyalcohols products differed

5) A. W. Van der Haar, Anleitung zum Nachweis, zur Trennung und Bestimmung der Monosaccharide und Aldehydsäuren, Berlin (1920).

6) M. Lambert and A. C. Neish, Can. J. Research, **B 28**, 83 (1950).

7) A. C. Neish, Analytical method for bacterial fermentation, 2nd rev. Research Council. Can., Praire Regional Lab., Saskatoon Saskatchewan N. R. C· No. 2952 (1952).

TABLE I. ANALYTICAL RESULTS OF THE FERMENTED BROTH

	18% NaCl medium*1)	30% glucose medium*2)
% of glucose consumed	9.16	23.04
rate of glucose consumption (%)	99.2	80.3
% of ethnol formed	0.09	2.09
rate of alcohol fermentation on glucose consumed	1.92	17.74
rate of other consumption on glucose consumed	98.08	82.26
total acid formed (%)	0.12	0.17
% of crude glycerol formed	3.938	6.303
yield of crude glycerol on glucose consumed	43.0%	27.4%
% of true glycerol formed	2.925	3.150
yield of true glycerol on glucose consumed	31.9%	13.7%
ratio of true glycerol to crude glycerol	0.75	0.50

*1) Initial glucose concentration is 9.23% and initial pH 4.4.
*2) Initial glucose concentration is 28.69% and initial pH 4.8.

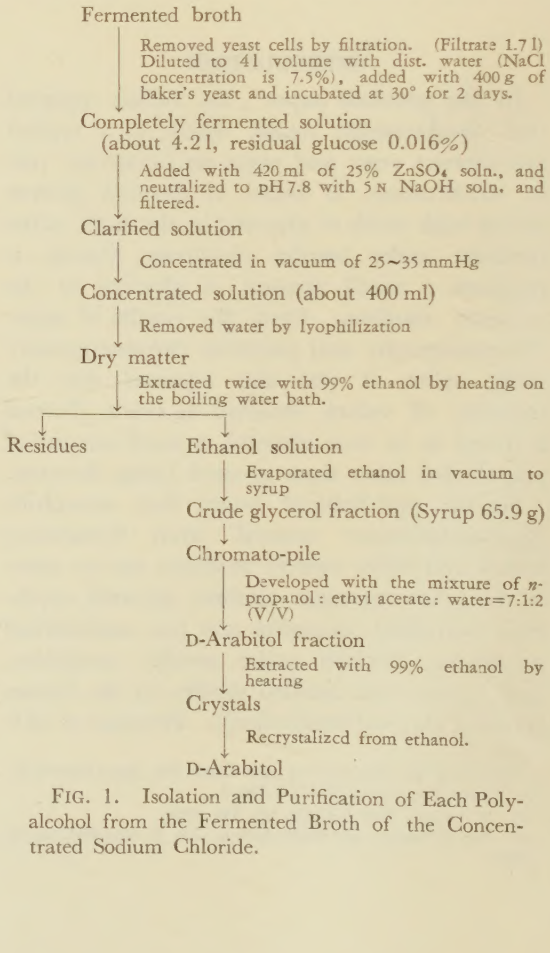

Fermented broth
Removed yeast cells by filtration. (Filtrate 1.7 l) Diluted to 4 l volume with dist. water (NaCl concentration is 7.5%), added with 400 g of baker's yeast and incubated at 30° for 2 days.
Completely fermented solution (about 4.2 l, residual glucose 0.016%)
Added with 420 ml of 25% $ZnSO_4$ soln., and neutralized to pH 7.8 with 5 N NaOH soln. and filtered.
Clarified solution
Concentrated in vacuum of 25~35 mmHg
Concentrated solution (about 400 ml)
Removed water by lyophilization
Dry matter
Extracted twice with 99% ethanol by heating on the boiling water bath.
Residues
Ethanol solution
Evaporated ethanol in vacuum to syrup
Crude glycerol fraction (Syrup 65.9 g)
Chromato-pile
Developed with the mixture of *n*-propanol: ethyl acetate: water=7:1:2 (V/V)
D-Arabitol fraction
Extracted with 99% ethanol by heating
Crystals
Recrystalized from ethanol.
D-Arabitol

FIG. 1. Isolation and Purification of Each Polyalcohol from the Fermented Broth of the Concentrated Sodium Chloride.

Fermented broth

↓ Removed yeast cells by filtration. (Filtrate 1.7 l) Diluted to 2 l volume with dist. water, added with 500 g of baker's yeast and incubated at 30° for 3 days.

Completely fermented solution (about 2 l, residual glucose 0.13%)

↓ Added with 230 ml of 25% $ZnSO_4$ soln., and neutralized to pH 7.8 with 5 N NaOH soln., and filtered.

Clarified soln.

↓ Concentrated in vacuum of 25~35 mmHg

Concentrated soln. (about 500 ml)

↓ Removed water by lyophilization

Dried materials

↓ Extracted twice with 99% ethanol by heating on the boiling water bath

Residues — Ethanol soln.

Ethanol soln. ↓ Extracted ethanol in vacuum to suitable volume and allow to stand in cool.

Crystalls — Ethanol soln.

Crystalls ↓ Recrystalized from ethanol

Crystalls of D-arabitol (33.9 g)

Ethanol soln. ↓ Evaporated ethanol in vacuum to syrup.

Crude Glycerol fraction (syrup. 58.3 g)

FIG. 2. Isolation and Purifications of Each Polyalcohol from the Fermented Broth of 30% Glucose.

in the two concentrated media. In order to find whether or not the same polyalcohols are formed in these two concentrated media, the isolation and crystallization of each polyalcohol from the fermented broth were conducted as follows.

Residual glucose, which interferes with isolation of polyalcohols from the fermented broth, was removed by fermentation with baker's yeast. In this case, it was also confirmed that baker's yeast could only produce glycerol and not other polyalcohols; when the 10% glucose medium was fermented by baker's yeasts, other polyalcohols except glycerol could not be detected on the paper chromatogram of the fermented broth. The method of paper chromatography was as follows: it was developed with the solvents composed of *n*-butanol, ethanol and water (4 : 1 : 4 by volume[8]) and of *n*-propanol, ethylacetate and water (7 : 1 : 2 by volume[9]), and the polyalcohols were detected by the method of Yoda[10].

The fermented solution which was made almost completely free from glucose, was clarified by the addition of a 1/10 volume of 25% zinc sulfate solution to the

8) R. H. Hackman and V. M. Trikojus, *Bicohem. J.*, **51**, 653 (1952).
9) J. Cerbulis, *Anal. Chem.*, **27**, 1400 (1955)
10) A. Yoda, *J. Chem. Soc. Japan*, **73**, 18 (1952)

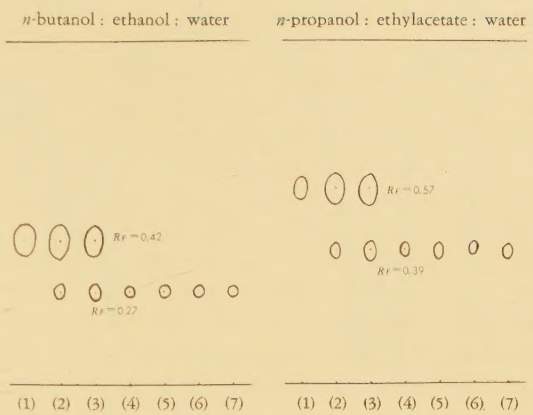
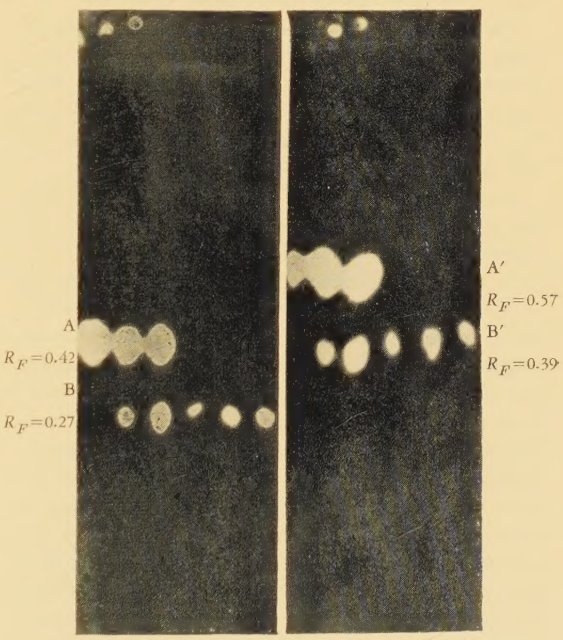


FIG. 3. Paper Chromatograms of Polyalcohol Isolated.

1) Authentic glycerol.
2) Crude glycerol fraction from 18% NaCl medium.
3) Crude glycerol fraction from 30% glucose medium.
4) Authentic adonitol.
5) Crystals obtained from 30% glucose medium.
6) Authentic D-arabitol.
7) Crystals obtained from 18% NaCl medium.



Photograph 1. Paperchromatograms of Polyalcohol Isolated.

1) Authentic glycerol.
2) Crude glycerol fraction from 18% NaCl medium.
3) Crude glycerol fraction from 30% glucose medium.
4) Authentic adonitol.
5) Crystals obtained from 30% glucose medium.
6) Authentic D-arabitol.

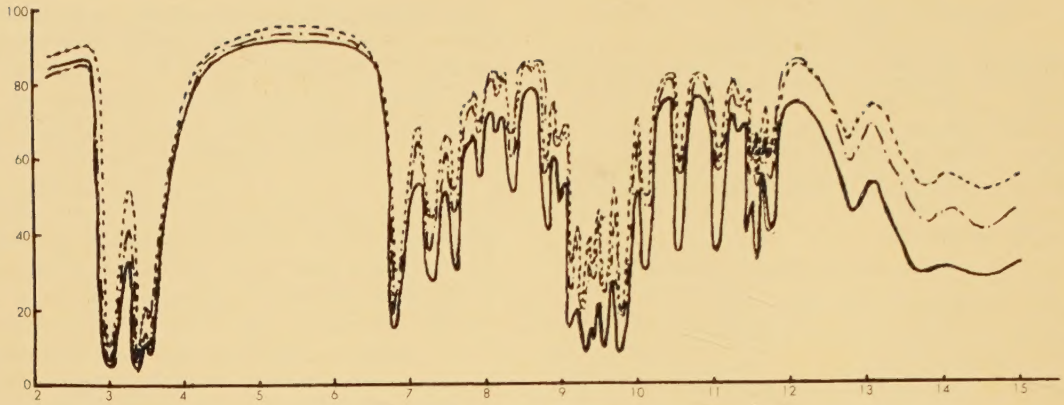


FIG. 4. Infra-red Spectra of D-Arabitol.
——: Crystals obtained from 18% NaCl medium.
- - - -: Crystals obtained from 30% glucose medium.
- • -: Authentic arabitol.

fermented solution and adjusting the pH value to 7.8 with 5 N NaOH solution, followed by filtration.

This clarified solution was concentrated by vacuum evaporation of 25~35 mmHg at 45~50°, and further completely dehydrated by lyophilization. The dried material was extracted twice with 99% ethanol by heating on a boiling water bath, and from this ethanol solution, D-arabitol was crystallized.

Procedures of isolation of glycerol and D-arabitol from the two concentrated media of sodium chloride and glucose are illustrated in Figs. 1 and 2.

By the above procedures, D-arabitol was only obtained in crystalline form from the concentrated glucose medium, but crystallization from the concentrated sodium chloride medium was not successful though the ethanol solution was concentrated to syrup. Then, it was shown by paper chromatography that this syrup contains both glycerol an D-arabitol (see Fig. 3 and Photograph 1). In this case, it seems that D-arabitol is dissolved in glycerol which is produced in a larger amount than D-arabitol. The application of chromatopile method was successful in the separation of these two polyalcohols each other. A stack of 800 sheets of the paper discs was clamped together, and developed with the solvent composed of *n*-propanol, ethylacetate and water (7 : 1 : 2 by volume). Then, the discs were separated and the constituents were tested for (R_F values were almost the same as those in paper chromatography). The discs of D-arabitol fraction were extracted with 99% ethanol by heating on a boiling water bath, and crystallization of D-arabitol from the ethanol solution was accomplished.

[IV] Identification of glycerol.

(1) Preparation of glycerol tribenzoate[11]: Place 100 mg of glycerol in a 25×200 mm test tube and add 0.5 ml of benzoyl chloride. Select a solid rubber stopper that securely fits the mouth of the tube. Add 5 ml of 10% sodium hydroxide solution and shake vigorously until the solid derivative separates out. Allow to stand in a cold bath, shaking the tube at intervals so that the lumps forming at the beginning break up into small granules; use a rod for this purpose if necessary. Add 5 ml of water, shake vigorously for a few minutes, and then filter. Wash twice with 5 ml of water and place on a drying disc. Glycerol tribenzoate is recrystallized from ligroins as needles, m.p. 71°. *Anal* Found: C, 71.18%; H, 5.47%. Calcd. for $C_{24}H_{22}O_6$: C, 71.27%; H, 4.99%.

This crystal did not depress the melting point on admixture with an authentic specimen which was prepared from special grade glycerol.

(2) Paper chromatography: As shown in Fig. 3 and Photograph 1, the spots of both glycerol fractions from the media of 18% NaCl and 30% glucose, agreed with those of the authentic glycerol.

[V] Identification of D-arabitol.

Three recrystallizations from ethanol, afforded prismatic crystals melting at 101°. This did not depress the melting point on admixture with an authentic specimen.

Two crystals from the media of 18% NaCl and 30% glucose, showed similar analytical results.

11) N. D. Cheronis and J. B. Entriken, Semimicro qualitative organic analysis, 2nd Ed., p. 383 (1957).

$[\alpha]_D^{20} = +8.28$ (C=9.78, in saturated borax solns.) *Anal.* Found: C, 39.40%; H, 7.98% (from 30% glucose medium); C, 40.48%; H, 7.90% (from 18% NaCl medium). Calcd. for $C_5H_{12}O_5$: D, 39.1%; H, 7.89%.

The infrared spectra of these samples were also found to be superimposable with that of the authentic specimen (Fig. 4).

As illustrated in Fig. 3, spots of these crystals agreed with those of the authentic D-arabitol.

Acknowledgement The author wishes to express his sincerest thanks to Emeritus Prof. K. Sakaguchi of University of Tokyo for his kind guidance and encouragement throughout this work. Great indebtness is also acknowledged to Prof. T. Asai, Prof. Y. Sumiki, Prof. K. Kitahara, Prof. K. Arima and Prof. Y. Ikeda for their valuable suggestions. Thanks are also due to prof. Y. Sumiki for the precious gift of D-arabitol, and to the Department of Agricultural Chemistray, University of Tokyo, for the infrared spectroanalysis and the microanalysis. The author also wishes to thank Dr. M. Mogi, the Director of Noda Institute for Scientific Research, for this encouragement and Mr. N. Saito for his willing help throughout the course of this work.

Studies on Osmophilic Yeasts*

Part VIII. Polyalcohol Production by Various Genera and Species of Yeasts

By Hiroshi ŌNISHI

From the results of investigation of the polyalcohol productivity of various genera and species of yeasts, the present author has now found that all the yeast strains tested show different individual characteristics in polyalcohol productivities and can be classified in the following six types: (1) solely producing glycerol, (2) solely producing erythritol, (3) producing only D-arabitol, (4) producing erythritol and D-arabitol, (5) producing glycerol and D-arabitol, and (6) producing glycerol, erythritol and D-arabitol.

It was also found that *Pichia miso*, which was isolated from miso-paste and showed a high osmophilic property, could produce glycerol, erythritol and D-arabitol giving very high yields, 50~60%, of sugar consumed. It seems to be worthy of note that this yeast was able to completely dissimilate highly concentrated glucose, such as 30%, producing much polyalcohols as those state above.

INTRODUCTION

In the previous paper[1,2], the author has found that *Sacch. rouxii*, which is a typical salt-tolerant yeast and plays an important role in soy-brewing, produces polyalcohols rendering high yields under aerobic fermentation in the concentrated media of NaCl or sugar, and the polyalcohols produced were identified as glycerol and D-arabitol by isolation in crystalline form from the fermented broths.

Studies on the production of polyalcohols other than glycerol by yeasts have not yet been reported with the exception of the production by genus *Zygosaccharomyces* alone, which has been shown by Spencer and Sallans[3], and Peterson et al.[4]. Previous knowledges are not available for the correlation between the genera and species of yeasts and their polyalcohol productivities.

Here-upon, the author has conducted extensive studies on polyalcohol production by various genera and species of yeasts including many osmophilic yeasts, from which is was revealed that these yeasts are classified into six types according to their characteristic productivities of the three polyalcohols, glycerol, erythritol and D-arabitol. Also, it was found that *Pichia miso*, which was isolated from miso-pastes by Mogi[5], can dissimilate sugar almost complety in a concentrated glucose solution such as 30%, producing glycerol, erythritol and D-arabitol with a high yield of 50~60% of glucose consumed.

EXPERIMENTAL AND RESULTS

[I] Polyalcohol production by various genera and species of yeasts.

Cultural conditions and analytical methods:

Since all of the 156 strains tested were able to grow well in a 30% glucose medium, polyalcohol production

*Reprinted by permission of Agr. Chem. Soc. Japan from Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 2.

1) H. Onishi, This Bulletin, **23**, 359 (1959).

2) H. Onishi, This Bulletin, **24**, 126 (1960).

3) J. F. T. Spencer and H. R. Sallans, *Can. J. Microbiol.*, **2**, 72 (1956).

4) W. H. Peterson, W. F. Hendershot and G. J. Hajny, *App. Microbiol.*, **6**, 349 (1958).

5) M. Mogi, *J. Agr. Chem. Soc. Japan*, **15**, 921, 1023, 1221 (1939); **16**, 7 (1940).

TABLE I. PRODUCTION OF POLYALCOHOLS BY VARIOUS GENERA AND SPECIES OF YEASTS.

(1) *Saccharomyces*

strain	% of glucose consumed	% of polyalcohol produced (as glycerol)	yield of polyalcohol on glucose consumed (as glycerol)	polyalcohol produced		
				D-arabitol	erythritol	glycerol
S. aggultianus Forma I	13.0	0.73	5.6%	—	—	++
S. aggultianus Forma II	8.7	0.64	7.3%	—	—	++
S. awamori	7.2	0.63	8.7%	—	—	++
S. anamensis	13.0	0.73	5.6%	—	—	++
S. cartilaginosus	2.0	0.31	15.5%	—	—	+
S. chevalieri	8.5	0.90	10.5%	—	—	++
S. exiguus	2.3	0.32	13.9%	—	—	+
S. fragilis	20.7	2.22	10.7%	++	—	+++
S. intermedius	4.0	0.45	11.2%	—	—	+
S. lindneri	10.1	0.78	7.7%	—	—	++
S. logos	7.5	0.27	3.6%	+	—	—
S. monasensis	4.6	0.68	14.7%	—	—	++
S. mongolicus	5.0	0.86	17.2%	—	—	++
S. paradoxus	9.7	0.94	9.6%	—	—	++
S. validus	2.8	0.37	13.2%	—	—	+
S. wilianus	8.0	0.78	9.7%	—	—	++
S. marxianus	11.1	1.59	14.3%	+	—	++
S. unisporus	2.4	0.52	21.6%	—	—	+
S. pasteurianus	3.3	0.47	14.2%	—	—	+
S. saké Kyokai 6	12.7	1.09	8.5%	—	—	++
S. carlsbergensis	7.9	0.70	8.8%	—	—	++
S. dairensis	5.2	0.46	8.8%	—	—	+
S. acidifaciens	19.7	5.13	26.1%	+++	—	+++
S. acidifaciens var. *halomembranis*	21.2	4.90	23.1%	+++	—	+++
S. manginii var. *miso*	9.3	0.82	8.8%	—	—	++

was examined by employing concentrated glucose medium composed of the following constituents: glucose 30%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCl_2 \cdot 2H_2O$ 0.01%, Casamino acid 0.4%, Yeast extract 0.1%, pH 4.8.

One drop of freshly cultured yeast suspension was inoculated into 400 ml shake-flasks containing 80 ml of the medium. The flasks were shaken on a reciprocal shaker operating at 120 r.p.m. with a stroke of 75 mm at 30°. After 10 days' incubation, the fermentation products were analysed according to the method of Neish[6]. At the same time, the fermented broth was subjected to paper chromatography to determine qualitatively the polyalcohol produced. Paper chromatograms were developed with a solvent composed of *n*-propanol, ethylacetate and water (7 : 1 : 2 by volume[7]) and the polyalcohols were detected according to the method of Yoda[8].

The results are shown in Table I.

As the results of this examination, some chracteristic differences in polyalcohol production could be recognized among various genera of yeasts; genus *Saccharomyces* which has been known as fermenting type, produced a relatively small quantity of polyalcohol, which mostly consisted of glycerol alone. (Typical strain: *Saccharomyces saké* and *Saccharomyces carlsbergensis*). In genus *Zygosaccharomyces*, a larger amount of polyalcohols was formed as compared with genus *Saccharomyces* and most of polyalcohols formed consisted of glycerol and D-arabitol. (Typical strains: *Saccharomyces rouxii* (*Zygosaccharomyces major*[1,2])).

In genus *Debaryomyces*, which is a no fermentation or

6) A. C. Neish, Analytical method for bacterial fermentation 2nd Rev., Natl. Research Council Can., Prairie Regional Lab., Saskatoon, Sask. N. R. C. No. 2952 (1952).

7) J. Cerbulis, *Anal. Chem.*, **27**, 1400 (1955).

8) A. Yoda, *J. Chem. Soc. Japan*, **73**, 18 (1952).

(2) *Zygosaccharomyces*

strain	% of glucose consumed	% of polyalcohol produced (as glycerol)	yield of polyalcohol on glucose consumed (as glycerol)	polyalcohol produced: D-arabitol	erythritol	glycerol
Z. bisporus	20.8	0.95	4.5%	++	−	++
Z. dairensis	12.1	0.62	5.1%	−	−	++
Z. fermentati	8.6	0.30	3.5%	−	−	−
Z. mellis-acidi	8.8	1.59	18.1%	++	−	++
Z. nadsonii	2.7	1.05	38.8%	++	−	++
Z. nukamiso	15.0	5.10	34.0%	++	−	++
Z. naniwaensis	20.3	1.33	6.5%	++	−	++
Z. vini	19.3	3.43	17.7%	++	−	++
Z. miso	20.3	2.16	10.5%	++	−	++
Z. amoeboidieus	12.2	1.93	15.9%	++	−	++
Z. citrus	14.2	3.45	24.3%	++	−	++
Z. felsineus	15.3	2.02	13.2%	++	−	++
Z. gracilis	13.7	3.03	22.1%	++	−	++
Z. gracilis var. *italicus*	21.2	3.40	15.9%	++	−	++
Z. mellis	7.3	2.15	29.4%	++	−	++
Z. nussbaumeri	2.9	1.12	38.6%	++	−	±
Z. polymorphus	11.0	2.29	20.8%	++	−	++
Z. rugosus	16.2	1.27	7.8%	++	−	±
Z. variabilis	17.9	2.09	11.7%	++	−	++
Z. halomembranis 433	5.7	0.95	16.7%	−	−	++
Z. halomembranis 471	18.6	3.65	19.0%	++	−	++

(3) *Hansenula*

strain	% of glucose consumed	% of polyalcohol produced (as glycerol)	yield of polyalcohol on glucose consumed (as glycerol)	polyalcohol produced: D-arabitol	erythritol	glycerol
H. saturnus	14.9	0.77	5.2%	++	−	−
H. suaveolens	1.6	0.14	8.7%	−	−	−
H. anomala	6.2	1.31	21.1%	±	−	+
H. anomala var. *productiva*	7.2	1.65	22.9%	+	−	+
H. mrakii	1.3	0.42	32.3%	−	−	−
H. schneggii	3.6	0.94	26.1%	+	−	+
H. miso β	20.5	4.94	24.8%	++	−	++
H. anomala var. *miso*	8.2	1.20	14.6%	+	−	++
H. miso β var. 1	9.9	2.38	24.0%	++	−	++
H. miso α	18.0	0.95	5.8%	+	−	++
Pseudohansenula peiping	16.7	1.94	9.7%	++	−	++

a very weak fermentation type, almost all of the strains produced only D-arabitol (Typical strain : *Debaryomyces saké*). Genus *Hansenula* formed one or two of D-arabitol and/or glycerol, and genus *Pichia* which, in a general, was an excellent producer of polyalcohols, produced two or three of glycerol, D-arabitol and erythritol. Especially, *Pichia miso*[5] was found to be the most superior strain for polyalcohol production among the 156 strains tested, giving a markedly high yield as 12.24 g of polyalcohol (calculated as glycerol) was formed from 28.6 g glucose dissimilated. *Pichia miso* which was isolated from miso-pastes and originally named by Mogi[5], was

(4) *Debaryomyces*

strain	% of glucose consumed	% of polyalcohol produced (as glycerol)	yield of polyalcohol on glucose consumed (as glycerol)	polyalcohol produced		
				D-arabitol	erythritol	glycerol
D. fabryi	10.5	0.22	2.1%	+	—	—
D. gruetzii	9.0	0.48	5.3%	++	—	—
D. hunderoi	9.6	0.49	5.1%	++	—	—
D. kloeckeri 7570	17.3	0.81	4.7%	++	—	—
D. kloeckeri 7050	8.1	0.29	3.5%	+	—	—
D. mandschuricus	10.1	0.35	3.4%	—	—	—
D. membranaefaciens	19.3	5.05	26.2%	++	—	++
D. membranaefaciens var. *Zingiberi*	7.7	0.85	11.1%	++	—	—
D. saké	10.0	2.05	20.5%	++	—	—
D. saké Froma I	9.0	0.48	5.3%	++	—	—
D. tyrocola	10.4	0.35	3.3%	++	—	—
D. tyrocola α	6.9	0.42	6.1%	±	—	—
D. tyrocola strain Okunuki	10.4	0.35	3.3%	++	—	—
D. kloeckri	6.4	0.66	10.3%	++	—	—
D. miso var. 1	8.4	1.84	21.9%	++	—	—
D. matruchoti	9.0	0.62	6.8%	+	—	—

(5) *Picha*

strain	% of glucose consumed	% of polyalcohol produced (as glycerol)	yield of polyalcohol on glucose consumed (as glycerol)	polyalcohol produced		
				D-arabitol	erythritol	glycerol
P. alcoholophila	23.1	5.43	23.5%	++	—	++
P. belgica	12.1	3.11	25.7%	++	—	++
P. farinosa	15.3	1.87	12.2%	++	—	++
P. hyalospora	16.6	1.74	10.4%	++	±	++
P. mandschurica (1)	16.3	1.90	11.7%	++	±	++
P. mandschurica (2)	16.1	2.21	13.7%	++	—	++
P. membranaefaciens	22.0	4.65	21.1%	++	—	++
P. polymorpha	21.0	4.72	22.4%	++	—	++
P. quercibus	8.9	0.18	2.0%	—	—	—
P. farinosa	14.3	1.22	8.5%	++	—	+
P. miso 7050	29.4	10.76	36.5%	++	—	++
P. miso 7061	28.1	9.11	32.4%	++	—	++
P. farinosa A 6	18.0	4.19	23.2%	++	—	++
P. miso H 52	28.6	12.24	42.4%	++	++	++
Zygopichia miso	17.0	1.28	7.5%	++	++	++
P. membranaefaciens	13.1	2.40	18.3%	++	—	++

renamed as *Pichia mogii*[9] or as *Pichia saké* form α[10] by subsequent investigators. In the anascosporogenous yeasts, various types of polyalcohol production were observed in strain by strain. Among those, *Torulopsis famate*[11] and *Candida polymorpha*[11], both of which wera isolated from soy-mashes at the initial stage of brewing and showed strong salt-tolerant and non-fermenting property,

9) I. Ohara and H. Nonomura, *J. Agr. Chem. Soc. Japan*, **28**, 122 (1954).

10) K. Kodama, *J. Fermentation Technol.*, **33**, 455 (1955).

11) H. Ōnishi, This Bulletin, **24**, 386 (1960)

(6) Other genera of yeast

strain	% of glucose consumed	% of polyalcohol produced (as glycerol)	yield of polyalcohol on glucose consumed (as glycerol)	polyalcohol produced		
				D-arabitol	erythritol	glycerol
Candida tropicalis	15.8	1.76	11.1%	++	−	+
C. lipolytica	5.2	0.36	6.9%	−	−	−
C. guilliermondii	9.5	1.40	14.7%	+	−	+
C. parapsilosis	11.9	1.23	10.3%	++	−	+
C. krusei	27.7	4.48	16.2%	++	−	+++
C. tropicalis A	20.3	1.48	7.2%	+++	−	+
C. tropicalis SA	12.1	0.91	7.5%	+++	−	−
C. arborea	6.1	0.70	11.5%	+++	−	−
Torulopsis halophilus	8.0	0.19	2.4%	−	−	−
Torulopsis nodaensis	8.2	0.42	5.1%	−	−	+
T. spherica	14.2	5.17	36.4%	−	−	+++
T. versatilis	13.9	6.81	48.8%	−	−	+++
T. osloensis	18.3	2.82	24.9%	+++	−	++
T. rubra	8.1	1.83	22.5%	−	−	++
T. uvae var. *miso*	7.2	0.73	10.1%	++	−	−
T. etchellsii	6.8	0	0	−	−	−
T. famata	13.7	4.11	30.6%	+++	++	−
C. polymorpha	7.5	1.67	22.2%	+++	++	−
Kloeckera africana	2.0	0.39	19.5%	−	−	+
Rhodotorula rosa	4.0	0.88	22.0%	++	−	+
Pseudomycoderma miso α	22.5	5.26	23.3%	+	−	+++
Pseudomycoderma miso β	6.5	1.96	30.1%	+++	−	+++
Monilia formosa	4.8	1.29	26.9%	+++	−	+++
Monilia vini	9.9	1.39	14.0%	+++	−	++
Monilia sitophila	16.8	1.90	11.3%	+++	−	++
Monilia sp.	5.1	1.56	30.5%	+++	−	+++
Mycotorula japonica	18.2	1.53	8.3%	+++	−	++
Mycoderma cerevisiae	2.3	0.27	11.6%	−	−	−
Geotricum candidum	2.1	0.62	29.5%	−	−	+
Trigonopsis variabilis	8.0	0.78	9.7%	−	++	−

(7) Miscellaneous yeast

strain	% of glucose consumed	% of polyalcohol produced (as glycerol)	yield of polyalcohol on glucose consumed (as glycerol)	polyalcohol produced		
				D-arabitol	erythritol	glycerol
S 3	28.9	9.28	32.1%	+++	−	+++
S 6	6.4	0.89	12.3%	+++	−	−
S 13	18.7	2.53	13.6%	+++	−	+
S 14	10.1	0.40	4.0%	++	−	−
S 15	6.9	0.14	2.0%	+	−	−
m 12	19.8	2.81	14.2%	+++	−	+++
m 13	12.2	1.00	8.2%	+++	−	−
7484	9.3	1.02	10.9%	−	−	++
E 1	20.2	2.73	13.5%	++	−	+++
E 3a	23.1	2.85	12.3%	+++	+	+++
E 3b	19.2	3.91	20.3%	+++	+	+++
E 3c	24.5	2.12	8.6%	+++	−	++
E 10	18.1	2.15	11.8%	++	−	++
E 11a	5.7	1.04	18.2%	+	−	+
E 11c	19.1	2.95	15.4%	++	−	+++
E g2	5.8	0.97	16.7%	−	−	++
E g3	19.0	1.83	9.5%	+++	−	+
E h	6.2	1.13	18.2%	++	−	+

Table II. Effect of Glucose Concentration in the Medium on Polyalcohol Production by *Pichia miso*.

glucose concentration of medium	% of glucose consumed	% of polyalcohol produced (as glycerol)	yield of polyalcohol on glucose consumed (as glycerol)	polyalcohol produced		
				D-arabitol	erythritol	glycerol
9.93%	9.82	1.84	18.7%	++	−	±
19.78%	19.19	6.13	31.9%	++	−	++
29.24%	27.65	11.91	43.1%	+++	++	+++
40.73%	23.15	9.80	42.3%	+++	++	+++
47.94%	15.52	5.20	33.5%	++	±	+++

Table III. Effect of Nitrogen Sources on Polyalcohol Production by *Pichia miso*.

nitrogen source and its concentration	% of glucose consumed	% of polyalcohol produced (as glycerol)	yield of polyalcohol on glucose consumed (as glycerol)	polyalcohol produced		
				D-arabitol	erythritol	glycerol
beef extract 0.4%	11.88	4.64	39.1%	+++	±	+++
corn steep lequor 0.4%	20.24	8.78	43.3%	+++	++	+++
polypeptone 0.4%	18.24	9.98	54.7%	+++	++	+++
yeast extract 0.4%	28.80	11.19	38.8%	+++	++	+++
ammonium lactate 0.2%	28.85	12.09	41.9%	+++	+++	+++
urea 0.05%	23.98	10.15	42.3%	++	++	+++
ammonium sulfate 0.2%	12.32	2.07	16.8%	++	−	++
ammonium chloride 0.2%	11.69	1.81	15.4%	+	−	++
ammonium sulfate 0.2% added with buffer	27.54	9.46	34.3%	+++	+	+++
ammonium chloride 0.2% added with buffer	23.32	7.79	33.4%	+++	+	+++

produced both of D-arabitol and erythritol in a relatively high yield. *Trigonopsis variabilis* produced only erythritol.

As a rule, osmphilic yeasts isolated from soy-mashes and miso-pastes were found to be excellent for polyalcohol production judging from the high yield of sugar consumed and the large absolute amount produced.

[II] Effects of various cultural conditions on polyalcohol production by *Pichia miso*.

Effects of various cultural conditions on polyalcohol production were observed employing *Pichia miso* H 52 which was proved to be the most excellent strain for polyalcohol production as shown in [I].

(1) Effect of glucose concentration.

As shown in Table II, the glucose concentration of 30~40% was most preferable for the production of polyalcohols. In case of 10% glucose, yield of polyalcohols produced was considerably low. As for the kinds of polyalcohols formed, D-arabitol was constantly produced independently of the glucose concentration, whereas erythritol could only be produced at glucose concentrations of 30~40%, and glycerol was scarcely produced in a 10% glucose medium.

(2) Effect of nitrogen source.

Both of ammonium N and amino N are suitable for the production of polyalcohols. Now, it should be noted that in a case wherein inorganic nitrogen sources such as $(NH_4)_2SO_4$ and NH_4Cl, are employed, the pH of the medium rapidly decreases to 2.0 or lower at the initial stage of fermentation and consequently, the yield of polyalcohols formed is reduced.

Therefore, when an inorganic ammonium salt is used as the nitrogen source, a buffer solution (e.g. K-citrate-citric acid buffer[12]) must be added to prevent tne depression of a pH of the medium during fermentation. (Table III)

(3) Polyalcohol production from various sugars.

As shown in Table IV, polyalcohols are remarkably produced from glucose, fructose or mannose, but scarcely formed from galactose, maltose and saccharose.

(4) Effect of aeration.

When 500 ml shake-flasks containing 30 to 80 ml of

12) H. Ohishi, This Bulletin, **21**, 143 (1957).

Table IV. Polyalcohol Production from Various Sugars by *Pichia miso*.

sugars	% of sugars consumed	% of polyalcohol produced (as glycerol)	yield of polyalcohol on sugars consumed (as glycerol)	polyalcohol produced		
				D-arabitol	erythritol	glycerol
30% glucose	28.6	12.24	42.4%	###	##	###
30% fructose	26.4	8.22	31.1%	###	+	###
30% mannose	19.1	6.49	33.9%	###	+	###
30% galactose	11.3	0.12	1.1%	—	—	—
30% maltose	0.3	0.04	13.3%	—	—	—
30% saccharose	10.1	0.14	1.3%	+	—	—

Table V. Effect of Aeration on Polyalcohol Production by *Pichia miso*.

cultural conditions	volume of medium per flask	% of glucose consumed	% of polyalcohol produced (as glycerol)	yield of polyalcohol on glucose consumed (as glycerol)	polyalcohol produced		
					D-arabitol	erythritol	glycerol
shaking	30 ml	27.98	12.96	46.3%	###	##	###
〃	50 ml	27.98	12.07	43.1%	###	##	###
〃	80 ml	27.65	11.91	35.3%	###	##	###
〃	120 ml	17.49	6.18	23.3%	###	±	###
〃	200 ml	11.71	2.73	23.3%	###	—	###
stationary	80 ml	5.49	1.94	35.3%	##	—	##

the medium were cultured under the above-stated conditions, polyalcohols were produced in good yields. In this case, the K_d value measured by the sodium sulfite method was 2.5×10^{-6} g mol/min.Atom. ml or higher.

However, when the flasks containing the medium exceeding 120 ml were cultured in the same conditions as mentioned above, the formation of polyalcohol was inferior to the above case. (In the case, K_d value is smaller than 1.7×10^{-6}). (Table V)

Table VI. Analtical Results of the Fermented Broth by *Pichia miso*.

% of glucose consumed	29.47%
pH	3.2
% of total polyalcohol formed (calculated as glycerol)	11.5
yield of total polyalcohol on glucose consumed (calculated as glycerol)	39.0%
% of D-arabitol formed	8.5
yield of D-arabitol on glucose consumed	28.8%
% of erythritol formed	0.6
yield of erythritol on glucose consumed	2.0%
% of glycerol formed	5.9
yield of glycerol on glucose consumed	20.0%
riboflavin formed μg/ml	2.1

[III] Isolation and purification of D-arabitol and erythritol from the fermented broth by *Pichia miso*.

(1) Analytical results of the fermented broth.

Analytical methods employed are the same as those outlined in the previous paper[1,2]. The values obtained by subtracting amounts of true glycerol from those of total polyalcohols (both calculated as glycerol), give the amount of D-arabitol and erythritol calculated as glycerol. D-Arabitol and erythritol were separately determined according to the method of Neish[6] by employing paper chromatography and subsequently eluting each of the spots with water.

The results are given in Table VI. The fermented broth showed yellow-brown colored fluorescene and the bioassay of this broth proved the formation of riboflavin, the amount of which is also given in Table VI.

(2) Isolation and purification of each polyalcohols from the fermented broth.

The methods of isolation of each polyalcohol were essentially the same as those in the previous paper[2], and the procedures are fully illustrated in Fig. 1.

From the ethanol solution, which was obtained by extracting completely dried materials of the broth, D-arabitol was obtained in crystals. However, the saparation of erythritol and glycerol from each other resulted

unsuccessful though the ethanol solution was concentrated to syrup. However, successful results were obtained when the chromato-pile method that is also effectively employed in the previous paper was applied. The discs of erythritol fraction obtained in chromato-pile method were extracted with 99% ethanol by heating on a boiling water bath, and erythritol could be crystallized from this ethanol solution.

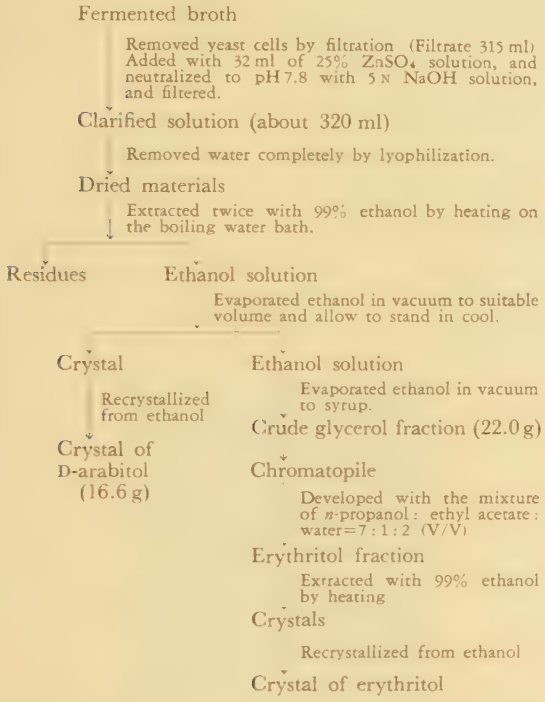

Fermented broth
Removed yeast cells by filtration (Filtrate 315 ml) Added with 32 ml of 25% $ZnSO_4$ solution, and neutralized to pH 7.8 with 5 N NaOH solution, and filtered.
Clarified solution (about 320 ml)
Removed water completely by lyophilization.
Dried materials
Extracted twice with 99% ethanol by heating on the boiling water bath.
Residues
Ethanol solution
Evaporated ethanol in vacuum to suitable volume and allow to stand in cool.
Crystal
Recrystallized from ethanol
Crystal of D-arabitol (16.6 g)
Ethanol solution
Evaporated ethanol in vacuum to syrup.
Crude glycerol fraction (22.0 g)
Chromatopile
Developed with the mixture of n-propanol : ethyl acetate : water=7 : 1 : 2 (V/V)
Erythritol fraction
Extracted with 99% ethanol by heating
Crystals
Recrystallized from ethanol
Crystal of erythritol

FIG. 1. Isolation and Purification of Polyalcohol from the Fermented Broth.

[IV] Identification of D-arabitol and erythritol.

Recrystallization from ethanol was carried out three times.

(1) D-Arabitol.

The D-arabitol thus obtained was prismatic crystal melting at 100.5~101.5°. This did not depress the melting point on admixture with an authentic specimen.

Anal. Found : C, 39.54%; H, 8.01%.

Calcd. for $C_5H_{12}O_5$: C, 39.41%; H, 7.89%.

$[\alpha]_D^{20}=+8.28$ (C=9.78 in saturated borax solns.)

The infrared spectra of this sample was also found to be superimposable with that of the authentic D-arabitol (Fig. 2). As illustrated in Fig. 4, the spot of this crystal agreed with that of the authentic D-arabitol.

(2) Erythritol.

The erythritol crystal obtained was tetragonal prisms melting at 119°. This did not depress the melting point on admixture with an authentic specimen.

Anal. Found : C, 39.78%; H, 7.98%.

Calcd. for $C_4H_{10}O_4$: C, 39.34%, H, 8.25%.

The infrared spectra of this sample was also found to be superimposable with that of the authentic erythritol (Fig. 3). As illustrated in Fig. 4, the spot of this crystal agreed with that of the authentic erythritol.

According to the above procedure, 13.0 g of glycerol, 1.2 g of erythritol and 19.4 g of D-arabitol were actually

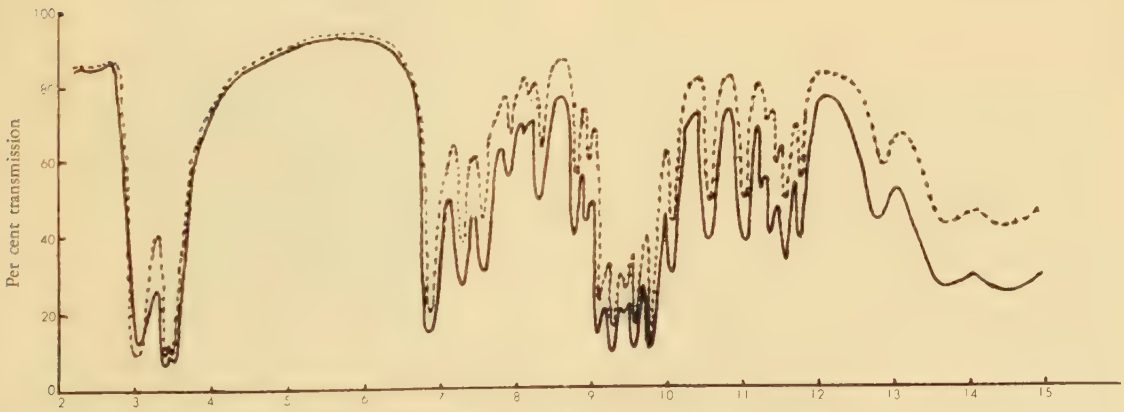


FIG. 2. Ihfra-red Spectra of the Crystal Obtained (D-Arabitol).

——: Crystal obtained
- - -: Authentic D-arabitol

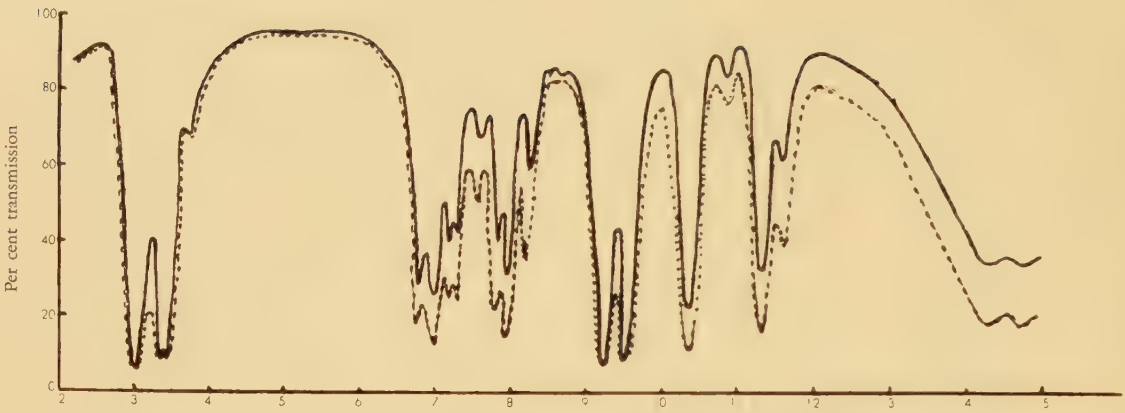


FIG. 3. Infra-red Spectra of the Crystal Obtained (Erythritol).

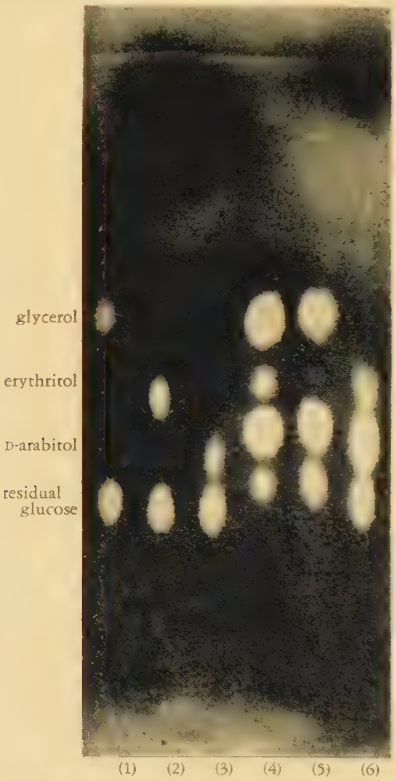
——: Crystal obtained
- - - -: Authentic erythritol

Fig. 4. Paperchromatogram of Crystals Obtained.

(1) Crystal obtained
(2) Authentic D-arabitol
(3) Crystal obtained
(4) Authentic erythritol

obtained from 315 ml of the fermented broth. When these values were compared with those shown in Table VI, recovery of glycerol, erythritol and D-arabitol were observed to be 69.7%, 63.4% and 73.3% respectively.

Paper chromatography of the fermentation products formed by 6 representative strains, which showed characteristic fermenting types for polyalcohol production, is illustrated in Photograph 1.



Photograph 1. Characteristic Fermenting Types for Polyalcohol Production by 6 Representative Strains.

(1) *Saccharomyces saké*, producing glycerol alone.
(2) *Trigonopsis variabilis*, producing erythritol alone.
(3) *Debaryomyces saké*, producing D-arabitol alone.
(4) *Pichia miso*, producing glycerol, erythritol and D-arabitol.
(5) *Saccharomyces rouxii*, producing glycerol and D-arabitol.
(6) *Torulopsis famata*, producing erythritol and D-arabitol.

Acknowledgement The author wishes to express his sincerest thanks to Emeritus Prof. K. Sakaguchi of University of Tokyo for his kind guidance and encouragement throughout this work. Great indebtness is also acknowledged to Prof. T. Asai and Prof. Y. Sumiki, Prof. K. Arima and Prof. Y. Ikeda for their valuable suggestions. Thanks are also due to Dr. W. Slooff of Gentraalbureau voor Schimmelcultures, Delft, Holland, Assist. Prof. H. Iizuka of Tokyo University, Mr. K. Tsubaki of the Nagao Institute, Tokyo, for kindly supplying the yeast strains, and to the Department of Agricultural Chemistry, University of Tokyo, for the infrared spectro-analysis and the microanalysis. The author also wishes to thank Dr. M. Mogi, the Director of Noda Institute for Scientific Research for his encouragement, and Mr. N. Saito for his willing help throughout the cource of this work.

Studies on Osmophilic Yeasts*

Part IX. Isolation of a New Obligate Halophilic Yeast and Some Consideration on Halophilism

By Hiroshi Ōnishi

The author has isolated an obligate halophilic yeast from old soy-mash and named it *Torulopsis halonitratophila nov. sp.* It is of particular interest that though this yeast shows obligate halophilic property at 30° it exhibits facultative halophilism when tested at a lower temperature such as 20°. This observation suggests that cultural temperature is one of the most important factors for halophilism.

INTRODUCTION

Flannery[1] proposed the following classification for microorganisms according to their growth responses in media containing salts.

I. Non-halophiles

A. Salt-sensitive: Grow only in media containing less than 2% salt.

B. Salt-tolerant: Grow best in media containing less than 2% salt, but are also able to grow in media containing salt at concentrations of more than 2%.

II. Halophiles

A. Facultative: Will grow in media containing salt at concentrations less than 2%, but grow best in media containing more than 2%.

B. Obligate: Grow only in media containing salt at concentrations exceeding 2%.

As far as bacteria are concerned, a number of microorganisms which are obligately halophilic have been recognized[2,3]. However, no obligate halophilism has yet been found in yeast, only salt-tolerant types being so far described.

In the present paper, the isolation of an obligate halophilic yeast from old soy-mash will be described. This yeast has been named *Torulopsis halonitratophila nov. sp.* according to taxonomic studies. Conditions affecting halophilism were also examined, and it was revealed that incubation temperature is the most important factor.

FXPERIMENTAL AND RESULTS

[I] Isolation of Yeast from Soy-mash.

An appropriate dilution of a 12 month-old say mash was plated on koji agar containing 18% NaCl. From colonies appearing on the plates after 10 days' incubation at 30°C, a strain of yeast M 3 was obtained. Subculture of the yeast was purified through single colony isolation on koji agar containing 18% NaCl.

[II] Halophilic Property of the Yeast Strain M 3.

As this yeast could originally not grow in an ordinary koji extract at 30°C, it seemed that this yeast required the addition of sodium chloride in the medium for growth. In order to acclimatize the yeast in the absence of salt, successive subcultures were carried out in media containing salt of a gradually decreasing concentration. The first medium contained 18% NaCl and cells grown in this medium were inoculated in the second medium containing 15% NaCl. In each subculture, concentration of NaCl in the medium was 3% lower than concentration of the preceeding subculture. The final medium contained no NaCl. It was ovserved that this yeast is able to grow in media containing more than 6% NaCl. In the 3% NaCl medium, however, growth

*Reprinted by premission of Agr. Chem. Soc. Japan from Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 3.

1) W. L. Flannery, *Bact. Rev.*, **20**, 49 (1956).

2) W. L. Flannery, R. N. Doetsch and P. A. Hansen, *J. Bact.*, **64**, 713 (1952).

3) J. Robinson and N. E. Gibbons, *Can. J. Botany*, **30**, 147 (1952).

was observed after a markedly long time lag; in the NaCl-free medium, no growth was observed even after incubation for a long period. It is of particular interest that this yeast showed such an obligate halophilic property.

[III] Growth Curues of the Yeast Strain M 3.

The basal medium used was as follows; glucose 10%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCl_2 \cdot 2H_2O$ 0.01%, NaCl 0.01%, casamino acid 0.4%, yeast extract 0.1%. Sodium chloride was added to the medium at 6 different concentrations. One loopful of the yeast cells taken from the culture on 6% NaCl koji agar after 7 days' incubation at 30°C was suspended in sterile physiological saline, then one drop of this suspension was inoculated to each medium and incubation was carried out at 30°C. The growth of yeast was measured turbidimetrically with a photoelectric spectrophotometer at 660 mμ.

The growth curves of this yeast in media at various concentrations of NaCl at 30°C are illustrated in Fig. 1. The optimum concentration of NaCl for growth of this yeast was found to be about 9~13%.

FIG. 1. Growth Curves at 30°C of the Yeast Strain M 3 in Media of Various NaCl Concentrations.

NaCl 0%
NaCl 3%
NaCl 6%
● NaCl 9%
▲ NaCl 13%
◑ NaCl 18%

In the previous paper[4], it was reported that *Saccharomyces rouxii*, which being a typical salt-tolerant type displays an important role in soy-brewing, is able to grow at a high temperature such as 40°C in media with a high concentration of salts or sugars, although the yeast failed to grow in the ordinary plain medium at the same temperature. In other words, *Sacch. rouxii* was transformed into apparently an obligate halophilic type when tested at a higher temperature as 40°C, but showed non-halophilic salt-tolerant property at 30°C. This fact suggests that cultural temperature may be the most important factor for microbial halophilism.

The growth of the yeast strain M 3 was examined at a relatively low temperature such as 20°C in the media with various concentrations of NaCl. The results are illustrated in Fig. 2. This yeast was able to grow in a NaCl-free medium at 20°C but was not able to grow at 30°C. However, the yeast showed better growth in a saline medium than in a NaCl-free medium at 20°C. The fact soon became clear that this yeast has obligate halophilic property at 30°C but is transformed into facultative halophilus at a lower temperature such as 20°C.

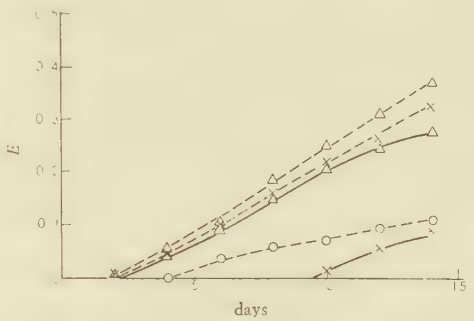


FIG. 2. Influences of Temperature on Groth of the Yeast Strain M 3 in the Ordinary Plain and the Salt-containing Media.

○: NaCl 0%
×: NaCl 3%
△: NaCl 6%
Solid line: 30°C
Broken line: 20°C

[IV] Taxonomic Studies on Yeast strain M 3.

The methods employed and key to the classification referred here were similar to those outlined in the monograph by Lodder and Kreger-van Rij[5]. Minor modifications and additions as described in the previous report[6] were made.

The experiments were carried out at an optimum condition for growth of this yeast, i.e. it was cultivated at 30°C in 6% NaCl media. The results are shown in Table I. The results were consistent with those obtained from experiments at 20°C using NaCl-free media.

According to the characteristics shown in Table I, this

4) H. Onishi, This Bulletin, **23**, 351 (1959).

5) J. Lodder and N. J. W. Kreger-van Rij, The Yeasts, a Taxonomic Study (1952).

6) H. Onishi, This Bulletin, **21**, 151 (1957).

TABLE I. MORPHOLOGICAL, CULTURAL AND PHYSIOLOGICAL CHARACTERISTICS OF THE YEAST STRAIN M 3

Vegetative cells after 10 days' incubation in 6% NaCl koji extract, at 30°C: Cells are oval, ellipsoidal and usually measure (2.5~3) $\mu \times (4 \sim 5)\ \mu$

Growth on 6% NaCl Koji agar, after 2 weeks at 30°C: The streak culture is greyish brown, dull glistening, raised and almost smooth.

Slide culture on 6% NaCl potato agar: No pseudomycelium

Ascospore formation: Not found on 6% NaCl Gorodokawa agar and 6% NaCl "V-8" agar, observed over a three months-period

Pellicle formation { in NaCl-free koji extract at 20°C: no pellicle; in 6% NaCl koji extract at 30°C: no pellicle; in 18% NaCl koji extract at 30°C: no pellicle

Fermentation of sugars { Fermented: glucose (week); Unfermented: galactose, maltose, saccharose, lactose

Assimilation of sugars { Assimilated: glucose; Not assmilated: galactose, maltose saccharose, lactose

Assimilation of nitrate: Positive

Ethanol as sole source of carbon: No growth

Splitting of arbutin: Negative

yeast belongs to genus *Torulopsis* and resembles *Torulopsis glabrata* (*Anderson*) *Lodder et de Vries* or *Torulopsis inconspicua Lodder et Kreger-van Rij* in respect of its abilities to ferment and assimilate sugars. The present yeast, however, is distinguished from those species upon judgement in terms of nitrate assimilation. In addition to this difference, the present yeast showed obligate halophilic property at 30°C and facultative halophilism at 20°C as described above.

From these characteristics, the present author regarded the strain M 3 as a new species and named it *Torulopsis halonitratophila nov. sp.*

[V] Influences of the Energy Sources and Sodium Chloride on Viability of *Torulopsis halonitratophila* at 20°C and 30°C.

The cells harvested from the fresh culture in 6% NaCl koji extract of *T. halonitrophila* were washed thoroughly and suspended in the following four types of media; ① pH 4.8 M/15 phosphate buffer; ② NaCl 6% in pH 4.8 M/15 phosphate buffer; ③ glucose 5% in pH 4.8 M/15 phosphate buffer; ④ NaCl 6% and glucose 5% in pH 4.8 M/15 phosphate buffer. The suspensions were incubated at eitheir 20°C or 30°C.

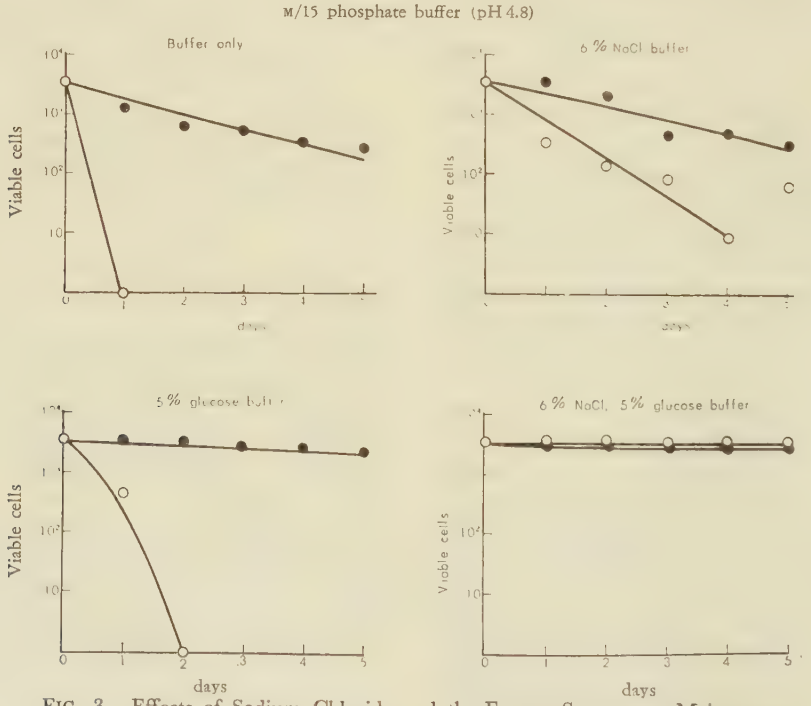


FIG. 3. Effects of Sodium Chloride and the Energy Sources on Maintenance of Cell-viability of *Torulopsis halonitratophila* at 20°C and 30°C. (1)
○: 30°C, ●: 20°C

Viability of the yeast cells was determined by plating aliquots on 6% NaCl koji agar. The plates were incubated at 30°C.

The results shown in Fig. 3, concerning the loss of viability at 30°C demonstrate that the addition of either 6% NaCl or 5% glucose had no protective effect on the cells. At the simultaneous presence of both glucose and NaCl in the suspension, the yeast maintained its viability. However, when incubation was carried out at 20°C, cell viability was fully maintained without the addition of NaCl and glucose.

In the following experiments, the cells were harvested from NaCl-free koji extract culture incubated at 20°C. Four types of cell suspension were made as described above and incubated at 30°C. As illustrated in Fig. 4, a similar phenomenon as shown in Fig. 3 was observed regardless the differences in the method of preculture.

These phenomena concerning cell viability were essentially identical with those of *Sacch. rouxii* which were reported in the previous paper[4]. However, the characteristic difference between these two strains was that the present isolated yeast failed to grow in the salt-free medium when it was tested at 30°C, which was the suitable temperature for growth of *Sacch. rouxii* as well as the majority of yeasts.

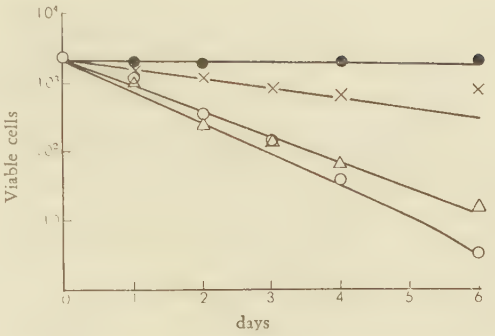


FIG. 4. Effects of Sodium Chloride and the Energy Source on Maintenance of Cell-viability of *Torulopsis halonitratophila* at 30°C. (2)

○: M/15 phosphate buffer
×: 6% NaCl phosphate buffer
△: 5% glucose phosphate buffer
●: 6% NaCl, 5% glucose phosphate buffer

[VI] Growth of *Torulopsis halonitratophila* in Various Hypertonic Media.

The methods of inoculation and cultivation were similar to those shown in [III]. As shown in Table II,

TABLE II. GRWOTH OF *Torulopsis halonitratophila* IN VARIOUS HYPERTONIC MEDIA

Media	10 days	30 days
Basal medium*	−	−
〃 added with 0.5 Mol KBr	−	−
〃 〃 1 Mol KBr	++	++
〃 〃 0.5 Mol LiCl	−	−
〃 〃 1 Mol LiCl	−	−
〃 〃 1 Mol NaCl	++	++
〃 〃 0.5 Mol Na_2SO_4	−	++
〃 〃 1 Mol Na_2SO_4	++	++
〃 〃 1 Mol $NaNO_3$	++	++
〃 〃 2 Mol $NaNO_3$	++	++
〃 〃 1 Mol KCl	++	++
〃 〃 2 Mol KCl	++	++
〃 〃 1 Mol KNO_3	+	++
〃 〃 2 Mol KNO_3	++	++
〃 〃 0.3 Mol $CaCl_2 \cdot 2H_2O$	±	±
〃 〃 0.5 Mol $MgSO_4 \cdot 7H_2O$	−	−
〃 〃 1 Mol $MgSO_4 \cdot 7H_2O$	−	−
〃 〃 20% glucose	+	++
〃 〃 40% 〃	+	+
〃 〃 60% ±	±	±

* Basal medium:

glucose	5%	KH_2PO_4	0.1%
$MgSO_4 \cdot 7H_2O$	0.05%	$CaCl_2 \cdot 2H_2O$	0.01%
casamino acid	0.4%	yeast ext.	0.1%

T. halonitratophila exhibits appreciable growth at 30°C in various media containing $NaNO_3$, Na_2SO_4, KBr, KCl, or KNO_3 instead of NaCl at high concentrations. The role of salts could be substituted with glucose at concentrations higher than 20 per cent.

[VII] Standard description of *Torulopsis halonitratophila nov. sp.*

Growth in 6% NaCl koji extract: After kept 10 days at 30°C, cells are oval to ellipsoidal, $(2.5\sim3)\mu\times(4\times5)\mu$. Only a sediment is formed.

Growth on 6% NaCl koji agar: After 2 weeks at 30°C, the streak culture is greyish brown, dull glistening, raised and almost smooth.

Slide culture on 6% NaCl potato ager: No pseudomycelium.

Ascospore formation: Not found on 6% NaCl Gorodokowa agar and 6% NaCl "V-8" agar, observed over a three month-period.

Fermentation: Glucose+(week) Maltose−
Galactose− Lactose−
Saccharose−

Sugar assimilation: Glucose+ Maltose−
Galactose− Loctose−
Saccharose−

Assimilation of potassium nitrate: Positive
Ethanol as the sole source of carbon: No growth
Splitting of arbutin: Absent
Halophilic progerty: Obligate halophilic at 30°C.
Facultative halophilic at 20°C.

DISCUSSION

Torulopsis inconspicua Lodder et Kreger-von Rij and *Torulopsis glabrate (Anderson) Lodder et de Vries*, which resembled the author's *Torulopsis halonitratophila*, were isolated from tannery liquid by Boidin and Abadie[7] and from concentrated orange juice by Recca and Mrak[8], respectively. These species have been found in concentrated solutions of salts or sugars. *T. halonitratophila* is distinguished from *T. inconspicua* and *T. glabrata* in respect of its ability to assimilate nitrate. It is of interest to note that *T. halonitratophila* does not form pellicles on any liquid media with or without NaCl in spite of its ability to assimilate nitrate. This property is considered to be a marked characteristic of most of salt-tolerant *Torulopsis* isolated from soy mash since many *Torulopsis sp.* showing such property besides *T. halonitratophila* have been isolated from soy mash by the present author[6,9].

As described previously, *T. halonitratophila* shows obligate halophilic property at 30°C and is transformed into a faculative halophilic type when tested at 20°C. Such special halophilic property has so far, not been found in yeasts. In the case of a halophilic bacteria, *Halobacterium halobium*, Christian[10] has reported that the requirement of sodium chloride for growth becomes less exacting at relatively low temperature.

As a conclusion of these observations, it should be emphasized that cultural temperature is the most important factors for halophilism.

Acknowledgement The author wishes to express his sincerest thanks to Emeritus Prof. K. Sakaguchi of University of Tokyo for his kind guidance and encouragement throughout this work. Great indebtness is also acknowledged to Prof. T. Asai and Prof. Y. Sumiki for their valuable suggestions. The author's thanks are also due to Dr. M. Mogi, the director of Noda Institute for Scientific Research, for his kind encouragement in this work.

7) J. Boidin and F. Abadie, *Bull. Soc. Mycol. France*, **70**, 353 (1954).
8) J. Recca and E. M. Mrak, *Food Technol.*, **6**, 450 (1952).
9) H. Onishi, This Bulletin, in press.
10) J. H. B. Christian, Dissertation, Cambridge (1956); cited from "Microbial Ecology" p. 102 (1957).

Studies on Osmophilic Yeasts*

Part X. Influence of the Environmental Factors on the Change of Microflora during the Ripening Process of Soy-mash

By Hiroshi Ônishi

Influences of concetrations of sodium chloride and pH values of soy mash on the changes of microflora during the ripening process were investigated. The proportions of various yeasts in the soy mash were proved to be changed with the above two environmental factors. And it was shown that growth sequence of yeasts in soy mash was controlled according to their pH sensitivities at the hypertonic condition. From young soy mash (3 days' old), the following yeasts were isolated: salt-tolerant type. . . .*Sccharomyces rouxii* Boutroux, *Torulopsis famata* (Harrison) Lodder et Kreger van Rij, *Candida polymorpha* Ohara et Nonomura, *Pichia farinosa* (Linder) Hansen, and *Trichosporon behrendii* Lodder et Kreger-van Rij; salt-sensitive type. . . .*Candida tropicalis* de Beurm., Gougerot et Vaucher) Ota and *Candida rugosa* (Anderson) Diddens et Lodder.

INTRODUCTION

Few papers have appeared which describe the ecological studies on the change of microflora during the ripening process of soy-mash.

Takahashi and Yukawa[1] stated that *Zygosacch. soya* was isolated from the young mash, whereas from the mature mash *Zygosacch. majar* was found. Kita[2] found that the species of yeasts isolated from the mash which had passed through the fermentation period in summer were quite different from those found in the immature mash before summer. Yoshii and Nakano[3] investigated the changes of microflora during the soy-brewing in terms of the plating counts of total yeasts, film-forming yeasts, total bacteria, bacilli and the lactic acid bacteria.

The soy mash differs significantly from other brew products in its high content of sodium chloride (about 18%) and high nitrogen concentrations (total nitrogen 1.0~1.5%). It is assumed that such a special environment has a remarkable effect on the microflora of the soy mash. The physiological aspects of osmophilic yeasts in the environment of high concentrations of sodium chloride were described previously by the author[4,5].

As far as the various soy mashes in the commercial scale (of the factory) were concerned, it was found that the pH values gradually shifted during the ripening process. The pH of the mash prepared in winter or spring was 6.0~6.6 and decreased to 4.4 in the case of fermentation period in summer. On the other hand, the initial pH value of the mash which was prepared in summer was 5.0~5.4 and rapidly became to 4.4.

When the mash was placed at 30°C which was suitable temperature for the growth of usual microorganisms, the pH decreased from 6.0~6.6 to below 5.0 in 15 days.

*Reprinted by permission of Agr. Chem. Soc. Japan from Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 4.

1) T. Takahashi and M. Yukawa, *Memoirs of the College of Agr., Tokyo Imp. Uuiv.*, **5**, 227 (1915).

2) G. Kita, *J. Chem. Ind. Tokyo, Japan*, **14**, 109 (1911).

3) H. Yoshii and M. Nakano, *J. Fermentation Technol.*, **34**, 361 (1956).

4) H. Ônishi, This Bulletin, **21**, 143 (1957).

5) H. Ônishi, This Bulletin, **23**. 351 (1959).

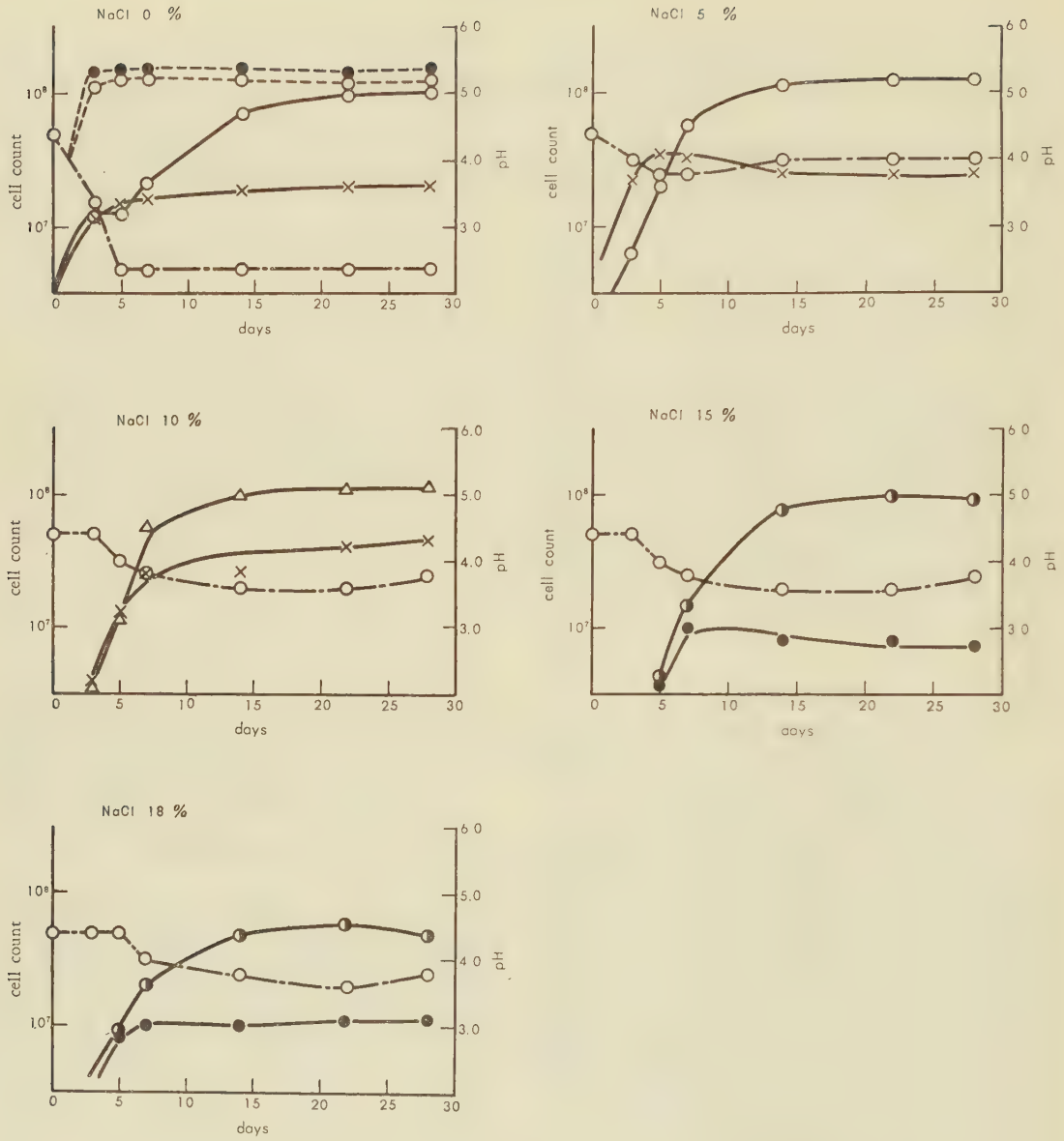


FIG. 1. Changes of Microflora of Young Soy Mash in Media of Various Concentrations of Sodium Chloride. (1) pH 4.4 koji extract.

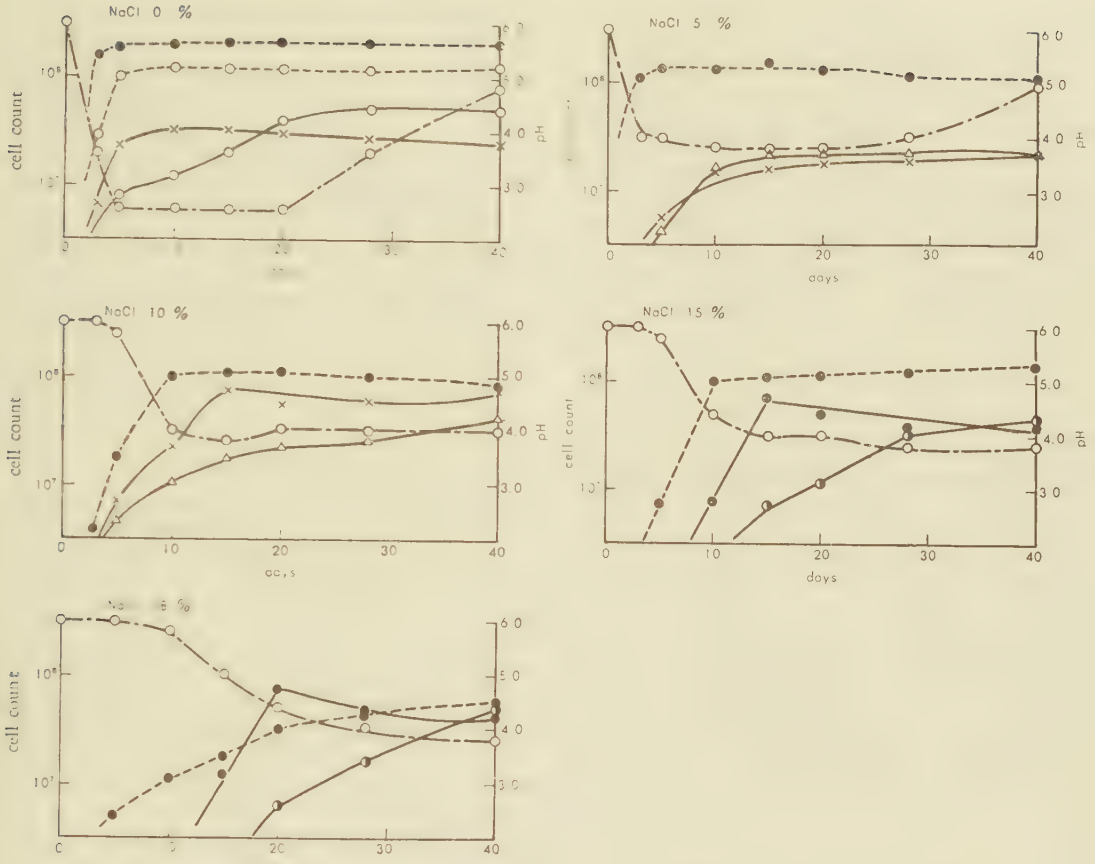


FIG. 2. Changes of Microflora of Young Soy Mash in Media of Various Concentration of Sodium Chloride. (2) pH 6.0 koji extract.

Bacteria { Rod : ○----○ / Cocci : ●----●
Yeast: Type I: ○—○ / Type II: ×—× / Type III: △—△ / Type IV: ●—● / Type V: ◑—◑
pH: ○—·—·—·—○

As described in the previous paper[4], the growth in the NaCl-free medium of *Sacch. rouxii*, which played an important role in ripening soy mash, was allowed at the wide range of pH (3.0~8.0), while in the 18% NaCl medium, the yeast grew only at pH 4.0~5.0. From these facts, it was estimated that an important factor controlling the change of microflora during the soy-brewing might be the pH value of the mash.

Various salt-sensitive yeasts and bacteria may exist in the soy mash at the initial stage of brining as well as the salt-tolerant micro-organisms. It may be interesting to see the interaction and the selection among these in the presence of various concentration of sodium chloride at different pH values.

In the present paper, the influences of various environmental factors on the change of microflora in the soy mash are described. However, the attention is focused to the pH value and

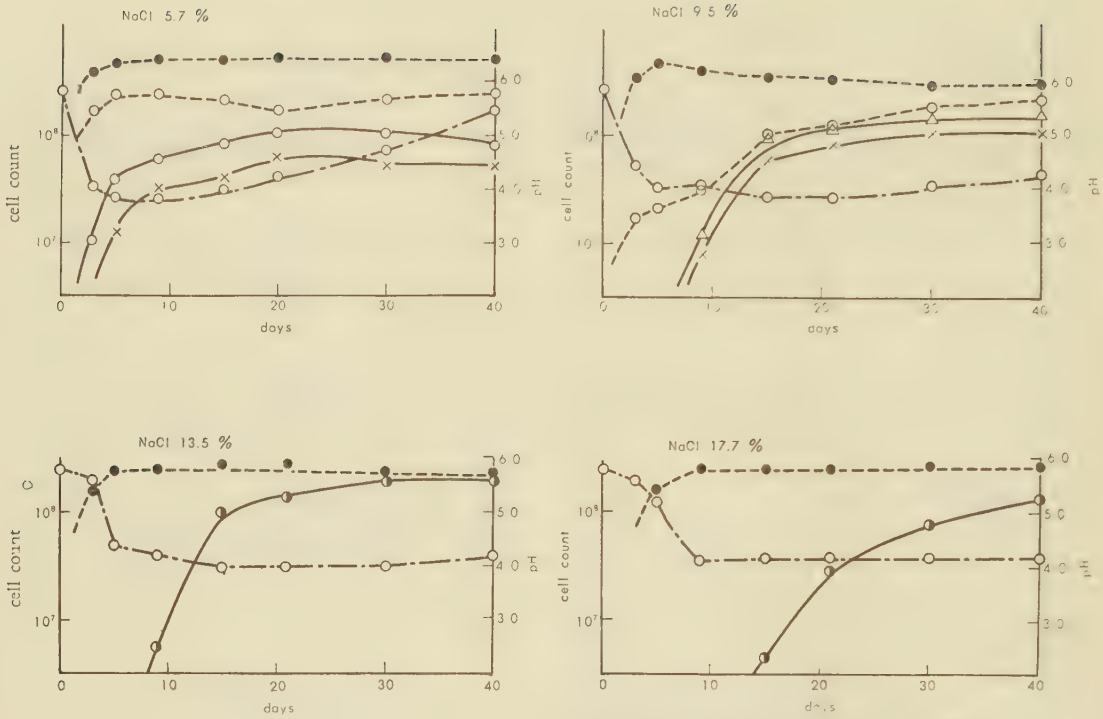


FIG. 3. Changes of Microflora in Soy Mashes of Various Concentrations of Sodium Chloride.

the salt concentration which have seemed important among the environmental factors.

MATERIALS AND METHODS

Investigation on the microflora was carried out by means of the routine techniques in the elective culture method and the enrichment method in soil microbiology[6].

Media: Koji extract (Balling 10°) were used which contained sodium chloride at various concentrations and of which the pH values were modified.

Materials: Samples of soy mashes were taken from the industrial tanks of the Noda Shoyu Co. One spoonful of the mash was suspended in 100 ml sterile saline prior to the examination.

Method of cultivation and observation: One to two drops of the mash suspension were inoculated into 100 ml of the koji-extract media and incubated at 30°C. Enumeration and identification of yeasts and bacteria were conducted under the microscope using the Thoma's Haemacytometer. The strains of yeasts were purified through single colony isolation and served for the taxonomic study.

6) S.A. Waksman, Soil Microbiology, p. 50 (1952).

EXPERIMENTALS

[I] Influences of the various concentrations of sodium chloride and of the different pH values on the changes of microflora in the soy mash.

The samples served for the following studies were taken from the young soy mash of 3 days' old after brining in August. This season was the fermentation period of the soy mash.

(1) Change of microflora in koji extract media of initial pH 4.4 in the presence of various concentrations of sodium chloride. (Fig. 1)

When the medium was free of NaCl, the pH of the

medium went down to 2.4 within 5 days. The shift of pH value was probably due to the growth of acid-forming cocci and rods. Approximately 10^8 of these bacteria were found in 1 ml of the medium. As for the yeasts, Type I and II were predominating and the former type propagated forming luxuriant pellicles on the whole surface of the medium. On the contrary, the presence of 5% or more NaCl in the medium did not permit the growth of bacteria and consequently the pH remained around 4.0. The slight decrease of pH values seemed to be due to the growth of yeasts. In NaCl-containing media, the different types of yeasts developed according to the concentrations of the salts: types I and II were predominating in the 5% NaCl medium, types I, II and III in 10% NaCl, and types IV and V in 15% or more NaCl.

(2) Change of microflora in koji extract medium of initial pH 6.0 in the presence of various concentrations of sodium chloride. (Fig. 2)

In the absence of NaCl, the growth of microorganisms and the fall of the pH value were similar to the results described in the preceeding section (1). However, the results were quite different when the medium contained NaCl. The media allowed the appreciable growth of acid-forming cocci in the early period of incubation, and the yeasts appeared later on. In the 18% NaCl medium, yeasts grew after pH of the medium fell below 5.0 in the following sequence: type IV appeared in the first place and then *Sacch. rouxii* of type V began to grow, the latter getting predominant finally.

In conclusion, rod-shaped bacteria were generally salt-sensitive and could not grow in a saline medium containing more than 10% NaCl, whereas cocci were considerably salt-tolerant. In the saline media, however, pH of the medium markedly influenced the growth of cocci. Namely, cocci could not grow in the saline medium of low pH value such as 4.4. (c. f.: Sakaguchi[7])

Yeasts were able to grow rapidly in the saline medium of pH 4.4, but when the pH was 6.0, the growth of yeasts could not start until the pH fell below 5.0. In the latter case, the yeasts of different types appeared in sequence as stated above.

(3) Change of microflora in the soy mashes of the various concentrations of sodium chloride. (Fig. 3)

Change of microflora in the soy mashes with the various NaCl concentrations was investigated. As shown in Fig. 3, cocci developed vigorously irrespective of the NaCl concentrations and consequently the pH of mashes went down. Rod-shaped bacteria did not grow in the soy mashes where the NaCl concentrations were more than 13.5%.

The sequences in the growth of various types of yeasts were similar to those described in the preceeding section (2).

(4) Analysis of the koji-broth fermented at various conditions described in the section (1) and (2).

It was assumed that the products by the different microflora would be dissimilar. Therefore, residual sugars and alcohol production in the modified koji extract media incubated with soy mashes were assayed after 35 days' incubation. The conditions of the experiments were described in the section (1) and (2). The amount of alcohol produced in the medium containing 10% or less NaCl was lesser than that expected on the basis of consumed sugar (Table I). The loss of alcohol might be due to the dissimilation by the pellicle-forming yeasts of type I and III. On the other hand, much alcohol was recovered in the 18% NaCl medium. The fermented medium containing 10% or less NaCl had characteristic odor of ethylacetate or mouldy smell which is not preferable for soy sauce. It should be noticed that NaCl at various concentrations controlling the change of microflora modifies the flavor and taste of soy sauce.

Table I. Analytical Data of the Fermented Broth in Experiment (1) and (2).

Initial pH of media	NaCl concentrations of media	Reducing sugars (as glucose) %	ethanol %
4.4	0%	0.91	0.05
	5	0.98	0.40
	10	0.96	0.01
	15	0.97	1.30
	18	1.00	1.19
	0%(Control)	8.77	0
6.0	0%	0.75	0.04
	5	0.63	0.66
	10	2.02	0.27
	15	1.07	0.95
	18	0.88	1.07
	0%(Control)	8.38	0.01

[II] Characteristics of Microflora in the Old Soy Mash.

The number of viable yeast cells in the soy mash of the fermentation period in summer were estimated to be 10^7 per ml of mash by plating method using koji agar containing 18% NaCl. In this period *Sacch. rouxii* was predominant.

In the older soy mash elapsed more than one year after brining, viable yeast cells were 10^4 per ml of mash and the predominant type of yeasts belonged to genus *Torulopsis*. This type of yeasts did not grow in the

7) K. Sakaguchi, This Bulletin, **23**, 22 (1959).

Table II. Morphological, Cultural and Physiological Characteristics of the Isolated Yeasts.

	Type I *Candida krusei* (Cast.) Berkhout	Type II *Candida tropicalis* (Cast.) Berkhout	Type III *Pichia farinosa* (Lindner) Hansen	Type IV *Torulopsis famata* (Harrison) Lodder et Kreger-van Rij
Vegetative cells (after 5 days' cultivation in koji extract at 30°C)	Cells are usually ellipsoidal to elongate. They measure about (3~5)×(6~12) μ. A dry white pellicle is formed.	Cells are usually round or oval, 5~10 μ. A sediment and a ring are formed, occasionally islets.	Cells are oval to cylindrical (4~6)×(4~8) μ. A wrinkled pellicle is formed.	Cells are small, oval, (2~4)×(4~7) μ. The cells are single, in pairs or clinging together in short chains. A sediment and a ring are formed, occasionally islets.
Growth on koji agar, at 30°C	After one month, the color of the streak culture is grey to yellow. The streak culture is dull, flat usually smooth or delicately wrinkled. The margin is sinuous with distinct pseudomycelium fringes.	After one month, color is cream to yellowish. The appearance of the streak culture is dull to somewhat glistening, folded. The margin is surrounded by mycelium	After one month, the streak culture is grey, dull, flat and wrinkled.	After one month, the streak culture is cream to grey, usually smooth, slightly glistening.
Slide culture on potato agar	Rich development of pseudomycelium.	Rich development of pseudomycelium	Primitive or no pseudomycelium	No pseudomycelium
Ascospore formation	not formed	not formed	Spores are formed after heterogamous conjugation. The spores are round, 2~4 per ascus.	Not formed
Fermentation of sugars: Fermented	glucose	glucose, galactose, maltose, saccharose	glucose, galactose	no fermentation
Fermentation of sugars: Unfermented	galactose, maltose, saccharose, lactose	lactose	maltose, saccharose, lactose	glucose, galactose, maltose, saccharose, lactose
Assimilation: Assimilated	glucose	glucose, galactose, maltose, saccharose	glucose, galactose	glucose, galactose, maltose, saccharose
Assimilation: Not assimilated	galactose, maltose, saccharose, lactose	lactose	maltose, saccharose, lactose	lactose
Assimilation of Nitrate	Negative	Negative	Negative	Negative
Growth in ethanol	growth	growth	growth	no growth
Splitting of arbutin	Negative	Slightly positive	Positive	Negative
Salt-tolerance	13% NaCl: growth 15% NaCl: no growth	13% NaCl: gowth 15% NaCl: no growth	18% NaCl: growth	18% NaCl: growth

	Type V	Type a	Type b	Type c	Type d
	Saccharomyces rouxii Boutroux	*Trichosporon behrendii* Lodder et Kreger-van Rij	*Candida rugosa* (Anderson) Diddens et Lodder	*Trichosporon cutaneum* (de Beurm., Gongerot et Vaucher) Ota	*Candida polymorpha* Ohara et Nonomura
Vegetative cells (after 5 days' cultivation in koji extract at 30°C)	Cells are round or short oval, single or in pairs. The cells usually measure $(5\sim8)\times(5\sim10)$ μ. A sediment and a ring are formed.	Cells are oval, $(3\sim5)\times(4\sim6)$ μ, some cells are elongated. Islets are formed.	Cells are oval or ellipsoidal, $(2\sim4)\times(4\sim8)$ μ. A thin pellicle is formed.	Cells are round, oval, ellipsoidal to elongated, $(4\sim5)\times(4\sim20)$ μ. A grey wrinkled pellicle is formed.	Cells are bottle shaped triangular or ellipsoidal $(3\sim4)\times(6\sim8)$ μ. Thin islets are formed.
Growth on koji agar, at 30°C	After 2 weeks, the streak culture is light cream colored, dull glistening, raised and almost smooth.	After one month, the streak culture is yellowish-white, tough, raised, wrinkled and hairy.	After one month, the streak culture is grey-yellowish, dull and wrinkled.	After one month the streak culture is yellowish, grey, raised, wrinkled, dry, dull, hairy.	After one month, the streak culture is grey-yellowish, glistening, smooth and flat.
Slide culture on potato agar	No pseudomycelium	True mycelium with blastospores and arthrospores is formed.	Well-developed pseudomycelium	True mycelium with blastospores and arthrospores is formed	Pseudomycelium is not much developed.
Ascospore formation		not formed	not formed	not formed	not formed
Fermentation of sugars: Fermented	glucose, maltose	glucose, galactose, maltose, saccharose, raffinose	no fermentation	no fermentation	no fermentation
Fermentation of sugars: Unfermented	galactose, saccharose, lactose	lactose	glucose, galactose, maltose, saccharose, lactose	glucose, galactose, maltose, sacchorose, lactose	glucose, galactose, maltose, saccharose, lactose
Assimilation: Assimilated	glucose, galactose, maltose	glucose, galactose, maltose, saccharose	glucose, galactose	glucose, galactose, saccharose, maltose	glucose, galactose, saccharose, maltose
Assimilation: Not assimilated	saccharose, lactose	lactose	maltose, saccharose, lactose	lactose	lactose
Assimilation of Nitrate	Negative	Negative	Negative	Negative	Negative
Growth in ethanol	no growth	growth	growth	no growth	no growth
Splitting of arbutin	Negative	Positive	Very slightly positive	Very slightly positive	Positive
Salt-tolerance	18% NaCl : growth	18% NaCl : growth	13% NaCl : growth 15% NaCl : no growth	13% NaCl: growth 15% NaCl: no growth	18% NaCl: growth

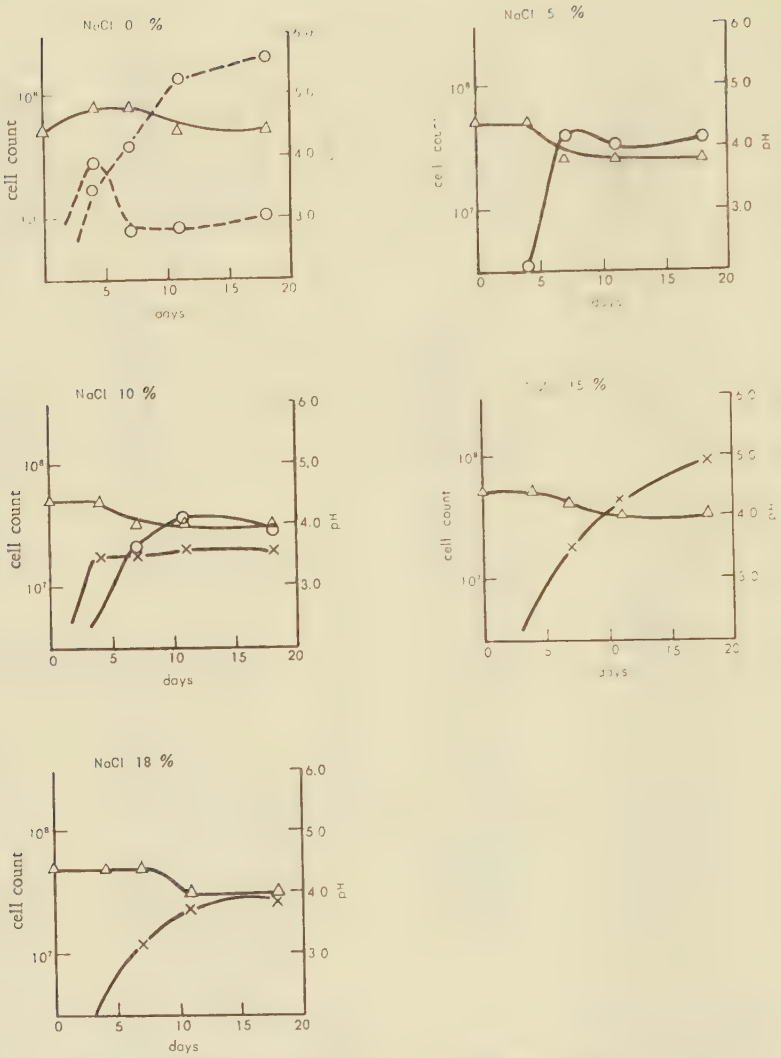


FIG. 4. Changes of Microflora of Old Soy Mash in Media of Various Concentrations of Sodium Chloride. (1) pH 4.4 koji extract.

Bacteria { Rod : ○----○ / Cocci : ●----● }
Yeast { Big cells : ○——○ / Small cells : ×——× }
pH : △——△

NaCl-free medium. When the old soy mash was inoculated into pH 4.4 or 6.4 koji extract containing NaCl at various concentrations, the microflora changed as illustrated in Figs. 4 and 5.

In the NaCl-free medium, both cocci and rod-shaped bacteria grew slowly whereas yeasts could not grow, and in the saline medium, vice versa. The yeasts grown in the saline medium were small and oval-shaped and could not grow in the NaCl-free medium. This yeast seemed to have obligate halophilic property. Microbiological properties of this yeasts were studied in detail and reported elsewhere[8].

8) H. Ônishi, This Bulletin, **24**, 226 (1960).

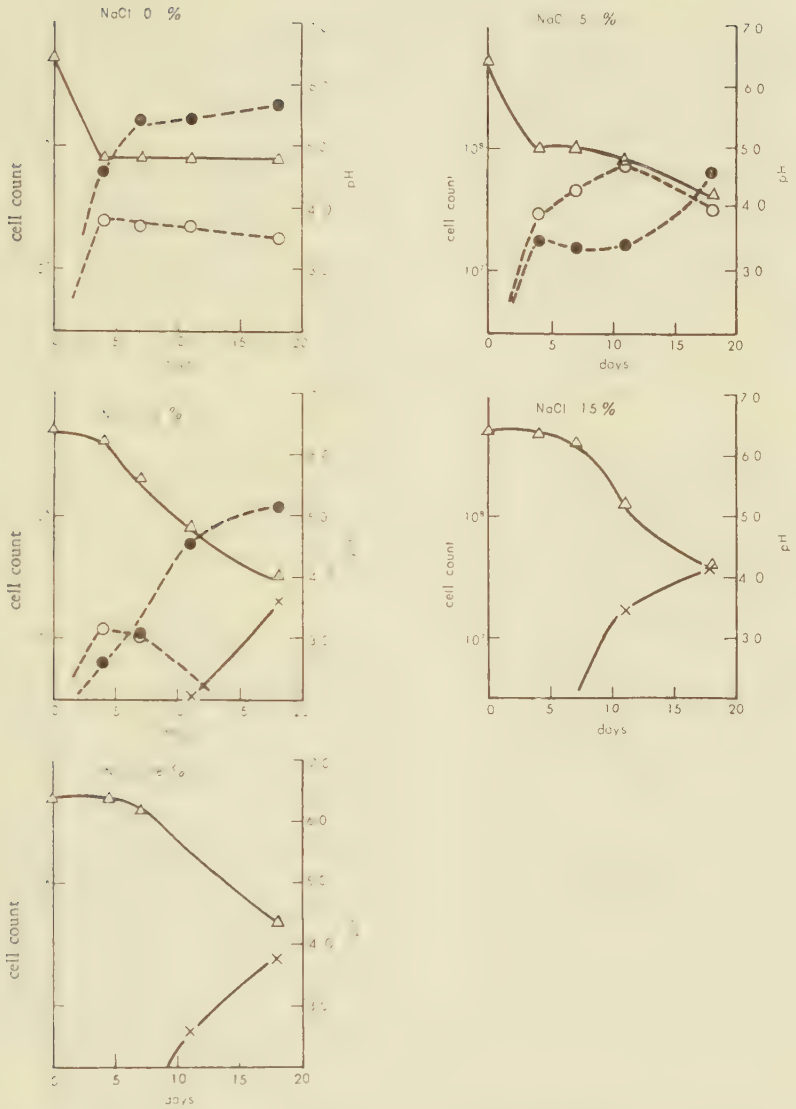


FIG. 5. Changes of Microflora Old Soy Mash in Media of Various Concentrations of Sodium Chloride. (2) pH 6.4 koji extract.

Bacteria { Rod : ○----○ / Cocci : ●----● }
Yeast { Big cells : ○——○ / Small cells : ×——× }
pH : △——△

Table III. Influences of the pH Values of Media on the Growth of Salt-tolerant Yeasts

Media \ yeasts / incubation period		*Torulopsis famata* 6 days' Incubation	*Torulopsis famata* 14 days' Incubation	*Candida polymorpha* 6 days	*Candida polymorpha* 14 days	*Sacch. rouxii* (isolated) 6 days	*Sacch. rouxii* (isolated) 14 days	*Sacch. rouxii* (stocked) 6 days	*Sacch. rouxii* (stocked) 14 days
NaCl-free	pH 3.0	−	±	−	−	+++	+++	+++	+++
〃	pH 4.0	+	++	+	+	+++	+++	+++	+++
〃	PH 5.0	++	+++	++	+++	+++	+++	+++	+++
〃	pH 6.2	++	+++	++	+++	+++	+++	+++	+++
〃	pH 7.0	++	+++	++	+++	+++	+++	+++	+++
NaCl 18%	pH 3.0	−	−	−	−	++	++	++	++
〃	pH 3.8	−	+	−	+	+++	+++	+++	+++
〃	pH 5.0	+	+++	±	++	++	+++	++	+++
〃	pH 6.0	±	+++	±	++	−	++	−	−
〃	pH 7.0	−	++	−	+	−	−	−	−

[III] Taxonomic Studies on the Isolated Yeasts. The yeasts arisen from the previous experiments, which were described in chapter [I], were purely isolated and classified according to Lodder and Kreger-van Rij[9]. Microbiological properties of these yeasts were shown in Table II. Types I∼V appeared always in soy mash and types a∼d were occasional.

[IV] Relationships between the Growth Sequence of Yeasts and pH Values in the Soy Mash. *Torulopsis famata* of type IV, *Candida polymorpha* of type d and *Saccharomyces rouxii* of type V had a highly salt-tolerant property and were found in the young soy mashes.

In the medium containing large amounts of NaCl, these yeasts grew in a constant sequence as stated above, i.e. first, type IV and then type V. It was examined whether this sequential growth was attributed to the pH-sensitivities of yeasts at the high concentration of NaCl. Koji extract media of various pH values and the media supplemented with 18% NaCl were employed for the study. One loopful of the yeast taken from the fresh culture on koji extract agar was suspended in sterile saline and one drop of this suspension was inoculated into each medium. The medium was incubated at 30°C. As shown in Table III, *Sacch. rouxii* (stock culture) and type V yeast were able to grow well in the wide range of pH 3∼7 when the media were NaCl-free, although in the 18% NaCl media, their growth was not allowed at the neutral side. On the other hand, type IV and d yeasts could not grow at pH 3.0 and their optimal growth, irrespective of the presence of NaCl, was exhibited at the relatively neutral conditions, pH 5∼7. These results may explain well the above described observation, that is, the pH value of the soy mash is an important factor which controls the sequence of yeast growth.

9) J. Lodder and M.J.W. Kreger-van Rij, The Yeasts, 1 taxonomic study (1952).

DISCUSSION

Yoshii and Nakano[8] reported that number of viable cells of lactic acid bacteria in soy mash decreased with the advance of ripening from 10^8 per ml at the initial stage of brining to 10^4∼10^5 per ml at the fermenting period. This fact is consistent with the author's observation that acid-forming cocci can not grow in a 18% NaCl medium at low pH such as 4.4 although the cocci grow well in the similar medium at pH 6.0. After acid-forming cocci have propagated in young soy mash of pH 6.0 lowering the pH below 5.0, yeasts start to grow vigorously.

Thus, principal roles of acid-forming cocci in young soy mash are to produce lactic acid at the initial stage of ripening of soy mash, and to lower the pH value of mash consequently bringing about a suitable environment for growth and fermentation of yeasts. Hori et al.[10] reported that principal organic acid in soy-sauce is lactic acid and that acetic or succinic acid is minor.

Yeast flora is clearly controlled by the concentrations of NaCl in the mash according to

10) S. Hori et al., *J. Agr. Chem. Soc. Japan*, **31**, 22 (1957).

the salt-tolerant properties of individials. As the saltsensitive yeasts are able to grow in the medium of 0~10% NaCl much faster than the salt-tolerant yeasts, the formers predominate over the latter in the mash containing relatively low concentrations of NaCl. In the medium of high concentrations of NaCl such as 18%, growth sequence of yeasts is controlled by pH values of the medium. When the brewing is initiated at pH 4.4, *Sacch. rouxii* can grow vigorously and quickly, while in the case where the pH is decreasing gradually from 6.0 to 5.0, the growth of *Torulopsis* preceed *Sacch. rouxii*, and the latter predominates finally playing an important role in soy ripening.

Acknowledgement The author wishes to express his sincerest thanks to Emeritus Prof. K. Sakaguchi of University of Tokyo for his kind guidance and encouragement throughout this work. Great indebtedness is also acknowledged to Prof. T. Asai and Prof. Y. Sumiki for their valuable suggestions. I also wish to thank Dr. M. Mogi, the Director of Noda Institute for Scientific Research, for his encouragement and Mr. A. Kaneko for his willing help at all stages of this work.

Metabolism of Aromatic Compounds by Microbes*

Part VI. Existence of Two Pathways, the Pathway *via* Protocatechuic Acid and the One *via* Gentisic Acid, in the Metabolism of *m*-Hydroxybenzoic Acid by Bacteria

By Sin-ichi Sugiyama, Keiji Yano, Kazuo Komagata, Masaji Kazuma and Kei Arima

The metabolic products of *m*-hydroxybenzoic acid formed by certain *Pseudomonas*, *Micrococcus*, and *Bacterium* strains which possess oxidizing ability of this acid were detected by paperchromatography. It was recognized that protocatechuic acid or gentisic acid are intermediary metabolites of *m*-hydroxybenzoic acid by these bacteria and the both acids are not detected in one cultural broth.

The authors reported the metabolic pathways of *m*-hydroxybenzoic acid by various bacteria using the technique of sequential induction method and presumed that this acid was metabolized either *via* gentistic acid (2,5-dihydroxybenzoic acid) or *via* protocatechuic acid (3,4-dihydroxybenzoic acid)[1].

In the previous reports, details in concern of the factors infulencing adaptive formation of the enzymes which oxidatively split *m*-hydroxybenzoic acid (*m*-HBA) and gentisic acid (GA) by *Pseudomonas ovalis* S-5 have been described[2,3]. On the metabolism of *m*-HBA by microbes, N. Walker[4] and B.S. Roof et al.[5] reported on their experiments conducted on specificities and strength of metabolic activities of several bacterial strains on this acid. However, the metabolic pathway of *m*-hydroxybenzoic acid has so for not been elucidated.

In this report detection of the metabolic products of *m*-HBA by the fifteen strains of bacteria which possess oxidizing ability of this acid by paperchromatography is described.

EXPERIMENTS

Biological: Fifteen strains of *m*-HBA metabolizing bacteria which were identified taxonomically in the previous report[6] were used.

Method of culture: Stationary or shaking culture at 30°C were made on synthetic medium containing *m*-HBA as the sole carbon source. Stationary culture was carried out in 1,000 ml of the medium in a 2,500 ml Fernbach flask and the cells were harvested after 4 days. Shaking culture was carried out in 100 ml of the medium in a 500 ml Sakaguchi flask using reciprocal shaker (130～140 r.p.m. 7 cm) and the cells were harvested after 16～18 hours.

Cultural medium.

Nitrogen source: As nitrogen source, three compounds such as $(NH_4)_2SO_4$, NH_4NO_3 and $(NH_4)H_2PO_4$, were tested for the accumulation of gentisic acid and protocatechuic acid and were proved to have no difference.

Influence of pH: Three levels of initial pH value such as 5.2, 7.2 and 8.5 were examined, and a shown in Table 1, pH 7.2 was proved to be best for the ac-

*Reprinted by premission of Agr. Chem. Soc. Japan from Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 2.

1) K. Arima, K. Komagata and N. Minoda, *J. Agr. Chem. Soc. Japan*, **28**, 629 (1954).

2) K. Arima, K. Komagata, S. Sugiyama, M. Kazama and K. Yano, This Bulletin, **19**, 51 (1955).

3) K. Arima, K. Komagata, M. Kazuma, S. Sugiyama and K. Yano, This bulletin, **19**, 61 (1955).

4) N. Walker and W. C. Evans, *Biochem. J.*, **52**, proc. XXXIII (1952).

5) B. S. Roof. T. J. Lannon and J. Turner, *Proc. Soc. Expt. Biol. Med.*, **84**, 38 (1953).

6) K. Arima, K. Komagata, S. Sugiyama and M. Kazama, *J. Agr. Chem. Soc. Japan*, **28**, (1954).

TABLE I. THE EFFECT OF pH ON PRODUCTION OF GENTISIC ACID OR PROTOCATECHUIC ACID BY SEVERAL BACTERIA

	pH value		U.V. ray*	Detection		pathway**
	Start.	After culture		$FeCl_3$	Diazo-reaction	
Micrococcus ureae S-4	5.2	5.2	−	−	+	G
	7.2	7.2	+	−	±	
	8.5	7.8	+	−	+	
Ps. ovalis S-5	5.2	5.4	−	−	+	G
	7.7	7.2	++	−	+	
	8.5	8.0	++	−	+	
Bact. sp. Ca-1	5.2	5.4	−	−	+	G
	7.2	6.6	±	−	+	
	8.5	7.6	−	−	+	
Ps. cruciviae P-8-2	5.2	5.6	−	−	±	P
	7.2	7.0	−	−	+	
	8.5	7.6	−	±	±	
Ps. dacunhae R-10-2	5.2	5.4	−	−	+	P
	7.2	6.6	−	±	+	
	8.5	7.8	−	−	+	
Ps. dacunhae G1-7	5.2	5.6	−	−	+	P
	7.2	6.6	−	+	+	
	8.5	7.8	−	−	+	

Surface culture at 30°C for 20 hrs. was made on synthetic medium (see text).
* Blue fluorescence
** The results of investigation by sequential induction method.
G—Gentisic acid pathway
P—Protocatechuic acid pathway

cumulation of GA. The composition of the synthetic medium which will be used in this experiment is as follows;

m-hydroxybenzoic acid	0.5 per cent
$(NH_4)_2SO_4$	0.1 〃
$MgSO_4 \cdot 7H_2O$	0.05 〃
KH_2PO_4	0.1 〃
pH	7.2

Preparation of samples for chromatography from cultural broth.

The procedure is as follows;

1,000 ml cultural broth

centrifugation at 3,000 r.p.m. 30 min. concentration in vacuum to 50 ml; acidified by H_2SO_4 to pH 2.0; ether extraction (cold) by a separation funnel, 3 times

Extraction by cold ether (Fraction 1)

Residual aqueous solution

continuous hot ether extraction for 40 hours

Fraction of hot ether extraction (Fraction 2)

Residue

Addition of 2, 4-dinitrophenylhydrazine (Fraction 3)

Preparation of samples of chromatography from incubation mixture.

The bacteria were cultured on bouillon-peptone broth which contained 1 per cent *m*-HBA, and was harvested by centrifugation after 18 hours. Ten g of the wet cells were suspended in 100 ml of M/15 phosphate buffer at pH 7.2 with 300 mg GA or PCA, and were iucubated at 30°C on the shaker. Sampling was carrid out at an interval of each 2 hours, and the production of ketoacids were examined as their 2, 4-dinitrophenylhydrazone.

Paperchromatography.

a) Filterpaper: The Tôyô filterpaper No. 50 (2×40 cm). Before development, filterpaper was saturated beforehand by the vapor of developing solvent in order to prevent the tailing of samples.

b) Developing solvents: After a survey was made for the purpose of detection of various intermediates, the following developing solvents were used;

1) Benzene: Acetic acid: Water (2 : 2 : 1)[7]

2) Butanol: Ethanol: $(NH_4)_2CO_3 \cdot NH_4OH$ buffer (4 : 1 : 2)[8,9]

7) H. G. Bray, W. U. Thorpe and K. White, *Biochem. J.*, **46**, 271 (1951).

8) D. Cavalini, N. Frontali and G. Toschi, *Nature*, **163**, 568 (1946).

9) M. E. Fewster and D. H. Hall, *Nature*, **168**, 78 (1951).

3) 3 per cent NH_4OH saturated butanol

4) Butanol : Formic acid : Water (4 : 1.5 : 1)

The solvents 1) and 2) were generally used for development of the aromatic compounds. The solvents 2) and 3) for development of 2, 4-dinitrophenylhydrazone of keto acids, and the solvent 4) for fatty acids. Each solvent was applied from the mixing after 3 to 4 days.

c) Development was carried out at 20°C by the ascending method.

d) Detection of compounds : The indicators to detect each compound are follows ;

1. *m*-hydroxybenzoic acid : the diazo reaction was used.

The first solution ; 1 per cent *p*-aminoacetophenone in 1 N H_2SO_4

The second solution ; 1 N HNO_2

After the development papers were dried at room temperature, the first and then the second solution were sprayed and the reddish orange spots appeared.

2. Gentisic acid : This was detected by the blue fluorescence when the paper was exposed in ultra violet rays in the dark room or by the brown spots by reaction with $FeCl_3$ (1 per cent $FeCl_3$ alcoholic solution was sprayed). Among these, the most convenient and sensitive indicator was found to be its own blue fluorescence.

3. Protocatechuic acid : The detection of the acid was either due to the diazo reaction similar to the case of *m*-HBA, or due to its dark green spot by reaction with $FeCl_3$. Usually, the dark green spot by spraying $FeCl_3$ was used.

4. Other phenolic compounds : These were mainly detected by the reaction with $FeCl_3$, and when the coloration was weak, alkalic solution was used as the indicator. Salicylic acid has a specific fluorescence under the ultra violet rays, and was detected by such characteristics.

5. Keto acids : The detection of keto acid was due to yellow spots of their 2, 4-dinitrophenylhydrazone.

6. Aliphatic acids : After development, the paper was put in a drier at 110°C for 10 min. Then bromcresolpurpule solution (0.16 per cent in 5 per cent alcohol adjusted to pH 8.0) was sprayed and yellow spots were revealed on the blue back ground.

RESULTS

R_F values of several related compounds.

R_F values of various phenolic acid and several other acids which were related with the terminal oxidants of these acids were estimated by different solvents. Table II shows the R_F values of these compounds developed by different solvents. These values are the average of several experiments and pyruvate was generously shown to have two spots when developed with the solvent 2.

TABLE II. R_F VALUES OF THE RELATED COMPOUNDS OF MONOHYDROXYBENZOIC ACIDS WITH VARIOUS SOLVENTS

	Solvent			
	I	II	III	IV
o-hydroxybenzoic acid	0.81	0.62		0.95
m-hydroxybenzoic acid	0.55	0.40		
p-hydroxybenzoic acid	0.72	0.34		
2, 3-dihydroxybenzoic acid	0.40	0.38		
β-resorcilic acid	0.27	0.40		
protocatechuic acid	0.12	0.18		0.85
gentisic acid	0.25	0.49		0.88
catechol	0.44	0.89		
resorcine	0.16	0.94		
hydroquinone	0.17	0.87		
2, 4-dinitrophenylhydrazone of levulic acid		0.48		
pyruvic acid		0.41, 0.61	0.34	
α-ketoglutaric acid		0.092	0.06	
oxaloacetic acid		0.14	0.07	
levulic acid				0.76
succinic acid	0.28			0.74
fumaric acid				0.81
maleic acid	0.18			0.47
citric acid	0.22			0.35

Solvent I ; Benzene 2 : Acetic acid 2 : Dist water 1
II ; *n*-Butanol 4 : Ethanol 1 : 1 N $(NH_4)_2CO_3$ buffer 2
III ; Butanol 3 per cent NH_3
IV ; *n*-Butanol 4 : Formic acid 1.5 : Dist water 1.

The detectable concentration of related compounds.

As shown in Table III, *m*-HBA was detectable from 3 gamma by the Diazo reaction, GA was detectable from 3 gamma by alkalic $FeCl_3$ solution, but from only 1 gamma by its specific blue fluourescence. PCA was detectable from 3 gamma with its green spot by $FeCl_3$ solution. Keto acids were detectable from 1 gamma in the form of their 2,4-dinitrophenylhydrazone. In the detection of fatty acids it was necessary to use at least 100 gamma of them for bromcresol-purple as an indicator.

Detection of aromatic compounds from stationary cultural broth.

R_F values obtained from the each Fraction 1 of the cultural broth of the several strains developed by the solvent 1 are shown in Table IV. Since the spots, of which the R_F value

Table III. The Detectable Concentration of Related Compounds by Several Reagents on Paperchromatography

	Concentration in spoting	Detection			
		$FeCl_3$	$FeCl_3$ $+NH_4OH$	U.V. ray	Diazo-reaction
gentisic acid	5	+	+	+	
	3	−	+	+	
	1	−	−	+	
protocatechuic acid	5	+	+	−	+
	3	+	+	−	+
	1	−	−	-	−
m-hydroxybenzoic acid	5				+
	3				+
	1				−

Developing solvent was the upper layer of a mixture of benzene 2, acetic acid 2, and water 1.

Table IV. R_F Values of the Intermediates of *m*-Hydroxybenzoic Acid in Fraction I in Stationary Culture

Strain	R_F Value	
Micrococcus ureae S-4	0.52	0.24
Ps. ovalis S-5	0.54	0.25
Bact. sp. Ca-1	0.57	0.27
Bact. sp. A-10-2		0.24
Micrococcus varians P-10-7	0.56	0.24
Ps. dacunhae G1-13	0.54	0.12
Ps. dacunhae G1-7	0.54	0.11
Ps. dacunhae R-10-2	0.55	0.11
Ps. dacunhae A-6-3	0.54	0.10
Ps. dacunhae A-7-2	0.54	0.12
Ps. crucivae P-8-2	0.52	0.12
m-hydroxybenzoic acid	0.55	
gentisic acid		0.25
protocatechuic acid		0.11

Developing solvent was the upper layer of a mixture of benzene 2, acetic acid 2, water 1.
Control were prepared as acetone solution.

was about 0.52 gave a negative reaction with $FeCl_3$ while by the Diazo reaction gave a clear reddish orange, this substance was considered to be *m*-HBA. The spot with a R_F value of 0.24~0.25 was proved to be GA, since their R_F and specific color reaction were the same as the synthetic samples GA was detected from the cultural broth of the strains S-4, S-5, A-10-2, and P-10-7. The strains G1-7, G1-13, R-10-2, A-6-3, A-7-2 and P-8-2 produced spots which were identifical with PCA.

The spot with a R_F value of 0.27 produced by strain Ca-1 was most probably GA due to its fluorescence and to the results of coloring reations, although the R_F value of 0.27 also corresponded to β-resorcicilic acid.

Detection of aromatic compounds from shaking cultural broth.

Table V shows the results of detection of the intermediate from shaking cultural broth. As well as in the stationary culture, GA was recognized in the cultural brothes of strains S-4, S-5, while PCA was detectable in those of strains G1-7, R-10-2 and P-8-2.

Summarizing the above results, as seen in Table VI, the same conclusion from the experiment with the sequential induction method was obtained. That is, *m*-HBA was oxidized *via* gentisic acid or protocatechuic acid according to bacterial strain, respectively.

Detection of keto acids.

As described above, the distinction between

Table V. R_F Values of the Intermediates of *m*-Hydroxybenzoic Acid in Fraction I in Shaking Culture

strain	R_F Value	
Micrococcus ureae S-4	0.51	0.25
Ps. ovalis S-5	0.52	0.24
Ps. dacunhae G1-7	0.57	0.12
Ps. dacunhae R-10-2	0.52	0.11

Developing solvent was the upper layer of a mixture of benzene 2, acetic acid 2, water 1.

TABLE VI. SUMMARIZING THE PRODUCTION OF GENTISIC ACID OR PROTOCATECHUIC ACID BY *m*-HYDROXYBENZOIC ACID ASSIMILATING BACTERIA

strain	Gentisic acid	Protocatechuic acid
Micrococcus ureae S-4	+	−
Ps. ovalis S-5	+	−
Bact. sp. Ca-1	+	−
Bact. sp. A-10-2	+	−
Micrococcus varians P-10-7	+	−
Ps. dacunhae G1-7	−	+
Ps. dacunhae G1-13	−	+
Ps. dacunhae R-10-2	−	+
Ps. dacunhae A-6-3	−	+
Ps. dacunhae A-7-2	−	+
Ps. cruciviae P-8-2	−	+

TABLE VII. R_F VALUES OF THE INTERMEDIATES OF *m*-HYDROXYBENZOIC ACID IN FRACTION III IN SHAKING CULTURE

Time (culture)	*Ps. ovalis* S-5		*Ps. dacunhae* G1-7	
	Spot I	Spot II	Spot I	Spot II
2	0.12	0.46		
4	0.16	0.49	0.14	
6	0.13	0.49	0.12	
8	0.13	0.49	0.13	
10	0.14	0.41	0.14	0.41
12	0.17	0.47	0.21	0.50
14	0.16	0.42	0.18	0.46
16	0.16	0.44	0.18	0.48
18	0.17	0.44	0.18	0.48
24	0.16	0.44	0.21	0.44

Developing solvent was the upper layer of a mixture of *n*-butanol 4, ethanol 1, water 2.

the strains having GA pathway and the other having the PCA pathway, was recognized. The detection of keto acids was carried out from incubation mixtures of *Ps. ovalis* S-5 and *Ps. dacunhae* G1-7. The former was oxidized *m*-HBA *via* GA, the latter oxidized *m*-HBA *via* PCA. An amount of 20 ml of samples was taken every 2 hours, and 2,4-dinitrophenylhydrazone was prepared and then developed in solvent 2. As seen in Table VII, different acids were detected depending upon the bacterial strain. In the case of *Ps. ovalis* the composition of acids was constant irrespective of the time of culture. Spot 1 of the former R_F value of 0.12 corresponds to α-ketoglutaric acid but assumed an orangish red color which is quite different from that of 2,4-dinitrophenylhydrazone of α-ketoglutaric acid. In the chromatogram of the mixture with the authentic α-ketoglutaric acid, the keto acid in question gave different spots hence the possibility that this keto acid is α-ketoglutaric acid was excluded. As for the spot 1 of *Ps. dacunhae*, from the R_F value it seemed to be 2,4-dinitrophenylhydrazone of α-ketoglutaric acid from paperchromatography mixture with the authentic samples. In the other case, spot 11 was not detectable between 2 to 8 hours' incubation, and was considered to be 2,4-dinitrophehylhydrazone of pyruvate.

DISCUSSION

These results do not clearly indicate successive steps of the metabolic pathway of *m*-HBA. However, they agreed with the assay of sequential induction method, which concluded that two metabolic pathways exist in the oxidation of *m*-HBA and is pathways are specific by bacterial strain. Namely, the existence of a common precursor is considered in the induced enzyme formation between analogous compounds, but against one inducer the bacterial metabolic activity differs between bacterial strains. R.Y. Stanier and N. Tsuchida[10] in bacterial metabolism of tryptophan, previously, domonstrated that there were two pathways, one the aromatic path *via* anthranilic acid, the other the quinoline path *via* quinolic acid, but both pathways did not exist together in one strain.

The production of lower keto acids are intermediary steps to terminal oxidation as well as the metabolism of aromatic compounds investigated by S. Dagley[11] and M. Isono[12]. An unknown keto acid produced by *Ps. ovalis* S-5 is considered to be a direct intermediate cleavaging benzene ring of GA. As will described in the

10) R. Y. Stanier and M. Tsuchida, *Biochem. J.*, **58**, 45 (1949).
11) S. Dagley, M. E. Fewster and F. C. Happold, *Biochem. J.*, **63**, 327 (1952).
12) M. Isono, *J. Agr. Chem. Soc. Japan*, **27**, 260 (1953).

latter report[13], the present authors assumed 7 carbons diketo-dicarboxylic acid, probably fumarylpyruvic acid, from the results obtained by manometrical experiment of gentisic acid oxidase. Therefore, considering the spot of its hydrazone not having appeared in developing Fraction 3 from cultural broth, it seems that metabolic decomposition of it had already been carried out or decomposition during prolonged hot ether extraction and other treatment had occurred.

SUMMARY

Oxidation of *m*-HBA by the eleven bacterial strains which were able to metabolize this compound as a sole source of carbon was carried out. The cultural broths were examined by paperchromatography for detection of intermediates.

Gentisic acid was identified in the culture filtrate of the five strains, while protocatechuic acid by the remaining six strains. Protocatechuic acid was not detected in all the cultural broth of bacteria which belong to the gentisic acid path type, and *vice versa*.

Strains which possess the gentisic acid path were *Pseudomonas ovalis* (two strains), *Micrococcus ureae*, and an unidentified *Bacterium* and a strain of *Aerobacter*. Strains which have the protocetechuic acid path were *Pseudomonas dacunhae* (five strains) and *Pseudomonas cruciviae*.

An unknown keto acid was detected as its 2,4-dinitrophenylhydrazone from the incubation mixture of the resting cell of *Pseudomonas ovalis* S-5 and gentisic acid. This new keto acid was most probably the direct intermediate after the cleavage of gentisic acid.

Acknowledgement The authors wish to express their sincere thanks to Prof. Emeritus K. Sakaguchi for his continous guidance throughout this work.

13) S. Sugiyama, K. Yano, H. Tanaka and K. Arima, This Bulletin, in press.

Metabolism of Aromatic Compounds by Microbes*

Part VII. Gentisic Acid Oxidase

By Shin-ici Sugiyama, Keiji Yano, Kazuo Komagata
and Kei Arima

A new enzyme which catalyzes an oxidative breakdown of gentisic acid with rupturing of the benzene ring was extracted from the *m*-hydroxybenzoic acid induced cells of *Pseudomonas ovalis* S-5 by several methods and named "Gentisic acid oxidase".

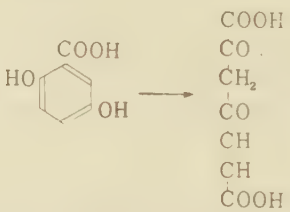
The enzyme was partially purified by various methods such as precipitation with ammonium sulfate, and ferrous ion was proved necessary for the activity of this enzyme.

Since, pyrocatechase was first discovered by M. Suda et al., in 1950, several other benzene ring splitting enzymes such as homogentisic acid oxidase[1,2], protocatechuic acid oxidase[3], 3-hydroxyanthranilic acid oxidase[4] have been reported. The present authors have investigated many bacteria isolated from soil and sewages which metabolize aromatic compounds, and in the bacterial metabolism of *m*-hydroxybenzoic acid (*m*-HBA), have demonstrated two pathways, the one the path *via* gentisic acid (GA) and the other *via* protocatechuic acid (PCA) from which it was found that the two pathways do not exist together in any bacterial strain.

Pseudomonas ovalis S-5 is one of the strains which oxidatively metabolize *m*-HBA *via* GA, accompanied by a cleavage of the benzene ring of GA. The authors have reported on the factors influencing enzyme formation in sequential induction methods[5,6].

The cell-free enzyme preparation which can oxidize GA was extracted from *m*-HBA induced cells of *Pseudomonas ovalis* S-5. This enzyme is a new one and requires ferrous ion as a sole cofactor and is named "Gentisic acid oxidase". As reported in the following paper, this β-diketodicarboxylic acid was isolated in a crystalline form from the enzyme reaction mixture and was identified as fumarylpyruvic acid. Further the initial intermediate maleylpyruvic acid has been also recognized spectrophotometrically by using a purified enzyme preparation[7].

The present paper is concerned with the extraction, partial purification and certain properties of gentisic acid oxidase, which catalyzes the following reaction:



MATERIALS AND METHODS

Biological: *Pseudomonas ovalis* S-5. The cells were grown in 100 ml of bouillon-peptone broth in a 500 ml Sakaguchi flask. Culture was continued for 18 hrs at 30°C with constant shaking. The cells were harvested by centrifugation, washed once with dist. water, and induced in M/15 phosphate buffer, pH 7.2 (wet cells 10 g/100 ml by M/100 *m*-HBA as an inducer[5,6].

*Reprinted by permission of Agr. Chem. Soc. Japan from Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 3.

1) M. Suda and Y. Takeda, *J. Biochem. Japan*, **37**, 375 (1959).
2) D. L. Crandall, *J. Biol. Chem.* **212**, 565 (1955).
3) R. Y. Stanier and J. L. Ingraham, *J. Biol. Chem.* **210**, 799 (1954).
4) R. W. Schayer and L. M. Henderson, *J. Biol. Chem.*, **195**, 657 (1952).
5) K. Arima, K. Komagata, S. Sugiyama, K. Kazuma and K. Yano, This Bulletin, **19**, 51 (1954).
6) K. Arima, K. Komagata, K. Kazuma, S. Sugiyama and K. Yano, This Bulletin, **19**, 61 (1954).
7) S. Sugiyama, K. Yano, H. Tanaka and K. Arima, This Bulletin, **24**, 255 (1960).

Determination and Chemicals: Enzymatic activity was determined by oxygen uptake using Warburg's manometer.

Contents of the Warburg vessel		
Main compartment	Enzyme solution	0.5 ml
	M/15 phosphate buffer pH 7.2	0.4
	Ferrous ion solution*	0.1
Center well	10 per cent KOH	0.1
Side arm	10^{-2} M sodium gentisate	0.3~0.5

* A mixture of ferrous sulfate and ascorbic acid (prepared at the final concentration of 10^{-3} M)

Specific activity is shown as Q_{O_2} (protein). Protein was determined by the micro-Kjeldahl method and by Folin-Ciocalteu method after precipitation by 10 per pent trichloracetic acid. GA used as the substrate was recrystallized before use.

Preparation crude enzyme extracts: Since the induced cells were completely inactivated by drying with cold acetone and lost their activity by vacuum dry treatment, the cell-free extract was obtained directly from the intact induce cells as shown in Table I.

TABLE I. METHODS FOR PRAPARATING CELL-FREE EXTRACTS

		Oxygen uptak
A)	From wet cells	
	1) Grinding with Alumina	++
	2) Grinding with Pyrex glass powder	++
	3) Grinding by Potter's homogenizer	+
	4) Ultra-sonic Vibration*	+
B)	From dry cells	
	1) Grinding with Alumina	+
	2) Grinding with Pyrex glass powder	+

* Vibrating condition of apparatus was 560 kc, 800 W

Although the enzyme activity could be demonstrated by each treatment, a considerable loss was always recognized except in case of grinding the intact cells with alumina. Extraction by sonic vibration was effective for large scale treatment, however, a considerable decrease of activity which might be caused by oxidation during the treatment was observed. Therefore, throughout this experiment the enzyme was extracted by the method of grinding with alumina.

The wet, packed induced cells were ground by hand for 30 min. with the same weight of levigated alumina. All preparative procedures were conducted from 0 to 4°C. The mixture was suspended in 10 ml of M/15 phosphate buffer (pH 7.4) per 10 g of wet cells.

The bulk of alumina was removed by low speed centrifugation and then the particulate fraction of the extracts was sedimented by refrigerating centrifugation for 30 min. at 10,000×g and discarded. A clear supernatant solution was obtained.

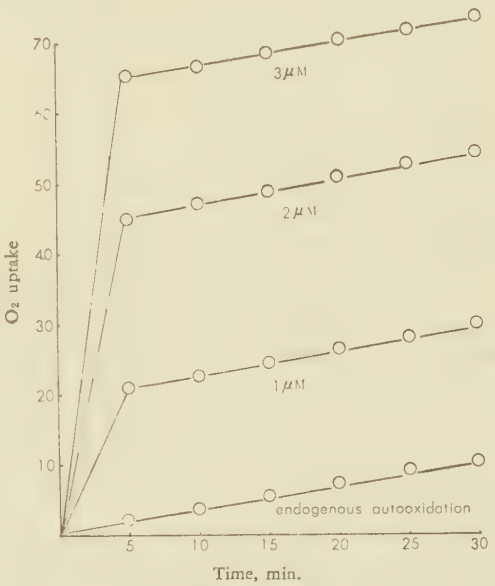


FIG. 1. O_2 Uptake for Gentisic Acid Oxidation by Crude Enzyme Preparation.

Several properties of crude extracts: Fig. 1 shows oxygen uptakes required for GA oxidation by crude extracts. One mole of oxygen was consumed per mole of GA. The oxidative activity of this enzyme preparation on PCA was demonstrated. However, *m*-HBA was not oxidized at all by this enzyme preparation. This preparation was stable at −20°C for one year, therefore, the enzyme solution were stored in a frozen state. Considerable losses of activity resulted after dialysis of the enzyme solution against dist. water or M/30 phosphate buffer pH 7.4 with agitation for 18 hrs, and almost complete losses of activity were shown against the stream water. From the above experiments it was suggested that the enzyme required some substance as a co-factor, such as metal ion which was rapidly removed from the apoenzyme. Table II shows the effect of various metal ions for the dialyzed enzyme. By the addition of 10^{-3} M ferrous ion, the inactivated preparation was almost restored to its original activity, though not completely, and was also not reactivated with ferric ion. When ascorbic acid was added with ferrous ion in order th maintain the level of ferrous ion, the effect

TABLE II. THE EFFECT OF METALIONS TO DIALYZED PREPARATION

Metal ions	Final concentration	O_2 uptake in 30 min.
Fe^{++}	10^{-3} M	47.63
Fe^{+++}	10^{-3}	5.99
Fe^{++}+V.C.	10^{-3}	62.31
Zn^{++}	10^{-3}	0
Mg^{++}	10^{-3}	0
$Mo(Na_2MoO_4)$	10^{-3}	0
Mn^{++}	10^{-3}	0
Co^{++}	10^{-3}	0
Cu^{++}	10^{-3}	0
Non add. (dialyzate)		0
Nondialysis preparation		63.75

Each ion adds from as sulfate except to Mo^{++}.

for the reactivation was greater than when they were used alone. Other metal ions can not replace ferrous ion as a co-factor.

The effect of other reducing agaents such as glutathione, cystein were shown in Fig. 2, where glutathione (10^{-3} M) gave the same effect as ascorbic acid. The role of ferrous ion on enzyme activity is described in a later paper[8].

As preliminary experiments for the enzyme purification, the following tests were carried out.

Heat treatment: Crude extracts adjusted at several pH values (5.4, 6.0, 7.2, 8.0, 9.2) were heated with stirring in a water bath at several temperature levels (50, 70, 100°C) for 3 to 5 minutes. Then they were immediately cooled by ice, and the resulted precipitate was removed by contrifugation and discarded. Activity of each supernatant was determined as shown in Fig. 3; the enzyme remained its activity at 50° for 3 min, but specific activity was decreased considerably. In respect to the relation between stability, this enzyme was most stable at pH 7.2 (see Fig. 3).

Adsorption by adsorbants: Suitable conditions were not obtained, because the crude extracts contained plenty of nucleic acids. The following adsorbants were tested for their adsorption effect. As enzyme activity is prominently decreased in the acid side any adsorption procedure was carried out in neutral and the slight alkaline side.

(1) **Calcium phosphate gel:** The technic employed here was simply to mix the adsorbent and solution together, separate the adsorbent, and elute the enzyme and activity of supernatant and eluted solution were determined[9]. As described in the later report[5] the adsorption effect of this gel was influenced by the existence of the phosphate ions in the enzyme preparation. Though the enzyme preparation extracten with phosphate buffer was not adsorpted with this gel in neutral pH ranges, the preparation extracted with trisaminomethane-HCl buffer was adsorpted strengthly, even if when they were treated at neutral pH ranges.

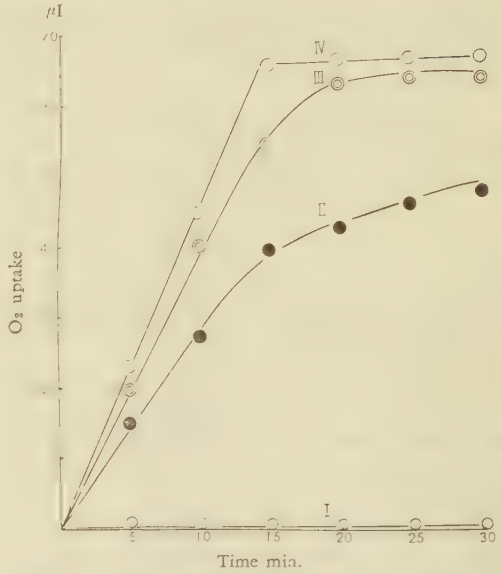


FIG. 2. The Effect of Glutathione of Cystein for Maintain the Level of Ferrous Ion.

I Dialyzed preparation: incubation of enzyme and substrate only
II With ferrous ion 2×10^{-3} M
III With ferrous ion and glutathione or crystein
IV Non-dialyzed preparation
The concentration of substrate was 3 μM in each case.

(2) **Hyflosupercel** Either in neutral pH or the acid side, the enzyme was not adsorpted by this adsorbent.

(3) **Active alumina** This was not a suitable adsorbent, as the pH value went up to 10.0 during the course of adsorption treatment.

Centrifugation: Crude extracts prepared by refrigerated centrifugation at $10,000\times g$ for 30 min, and the supernatants prepared by ultracentrifugation at $144,700\times g$ for 30 min maintained the whole of their activities.

From the results of the above experiments, purification of the enzyme was carried out.

To simplify the explanation, procedures of the enzyme purification is illustrated by the following scheme.

All the preparative procedures were carried out at 0

8) S. Sugiyama, K. Yano and K. Arima, This Bulletin, **24**, 249 (1960).

9) D. Keilin and E. F. *Biochem. J.*, **49**, **88** (1951).

TABLE III. PARTIAL PURIFICATION OF GENTISIC ACID OXIDASE

Fraction	Volume ml	Protein (mg) per ml	Protein (mg) total	Specific activity Q_{O_2} protein	Yield %
I	110	29.2	3,212	123	100
II	105	21.7	2,281	165	95.3
III	147	13.5	1,981	181	91.3
IV	20	10.2	204	1,125	57.9
V	20	9.7	193	1,150	56.2

~4°C. Throughout the course of purification, ferrous ion and ascorbic acid were added to each fraction as an enzyme protector.

Thus, the amber-colored and transport preparation Fraction V was obtained. Its specific activity was calculated to be Q_{O_2} (protein)=1150 (Table III), which is 8 times that of Fraction I.

DIAGRAM OF PURIFICATION PROCEDURE

Crude extracts (I)
| Treated with 0.5 per cent Hyflosupercel
Filtrates (II)
| 0.5 volume of protamine sulfate (2 per cent solution, pH 7.2 adjusted with ammonia) was added and ppt. was [illegible] by centrifugation, 3,000 r.p.m., 15 min.
Precipitates | Supernatants (III)
| fractionated with solid $(NH_4)_2SO_4$ 0.25~0.5 saturation
Precipitate dissolved in M/15 phosphate buffer pH 7.2 (IV) | Supernatants discarded
| Dialyzed overnight against dist. water (adjusted pH 7.2 with ammonia)
Dialysate
| Treated with a half-volume of Ca-phosphate gel and immediately centrifuged.
Gel discarded | Supernatants (V)

Properties of the partially purified enzyme (Fraction V): The oxygen uptake for the oxidation of GA was the same as that of crude extracts. One mole of oxygen per one mele of sodium GA was consumed and endogenous autooxidation was not detected. Since CO_2 was not evolved in the course of these reactions, as seen in Table IV, it is suggested that the oxidative decarboxylation had not occurred and a seven carbon intermediate is produced accompanied with benzene ring fission.

TABLE IV. CO_2 EVOLUTION BY OXIDATION OF GENTISIC ACID

		Sample	Non substrate
Total O_2 uptake	μl	56.60	+1.91
O_2 consumed calculated	mm	−22	+3
Observed pressure change due to add H_2SO_4 at start	mm	+13.1	+8.0
CO_2 evolution from phosphate buffer	μl	26.7	+17.45
CO_2 evolution calculated	mm	13.3	8.3
Observed pressure change due to add H_2SO_4 after 1 hr.	mm	−12	+9
Net pressure change due to oxidation culculated	mm	−3.3	−3.29
CO_2 produced by oxidation	μl	−6.612	−6.927
Net CO_2 produced by oxidation	μl	0.32	

Substrate concentration was 3 μM

TABLE V. CO_2 PRODUCTION IN $NaHCO_3$ SOLUTION WARBURG'S VESSEL CONTENT

Min.	Control O_2 uptake	CO_2 evolution in M/25 $NaHCO_3$
1	5.88	1.12
2	10.58	3.70
3	16.46	8.94
4	21.16	14.21
5	25.87	19.49
8	39.98	33.95
10	47.03	40.49
15	57.62	52.37
20	57.62	52.37
30	58.80	54.96

Control Usual Content	
M/25 $NaHCO_3$ solution	1.75 ml
Enzyme solution	0.25 ml
15% CO_2 in air.	gas phase
M/100 gentisate	0.3 ml

Table V shows carbon dioxide evolution during GA oxidation which was carried out in the CO_2-$NaHCO_3$

buffer. In this condition one mole of CO_2 per mole of GA oxidation was evolved. This means that one mole of carboxyl radical is produced in the reaction mixture by enzymatic oxidation of GA. As described in the previous report, the incubation mixture of induced cells with GA gave positive Rothera's reaction and produced 2, 4-dinitrophenylhydrazone. Furthermore, the reaction mixture of this partially purified enzyme reacted specifically with *o*-phenylenediamine. These facts supported the assumption that the direct product of GA oxidation accompanying the benzene ring cleavage is seven carbon β-diketodicarboxylic acid. As described in the appendix, the authors succeeded in the isolation and identification of fumarlypyruvic acid, and spectrophotometrically recognized maleylpyruvic acid which is the initial intermediate of GA metabolism of this *Pseudomonas*. The optimum substrate concentration is from 8 to 10 μM of GA, Km is 2×10^{-3} M.

Optimum pH: As seen in Fis. 4 the pH optimum of this enzyme preparation lies at pH 8.0. The addition of ferrous ion and ascorbic acid can recover enzyme activity at a low pH adjusted with 1 N acetic acid, similar to the case of homogentisic acid oxidase. From this, it is suggested that ferrous ion plays a role in stabilization of the enzyme.

Specificity: Several phenolic compounds were tested

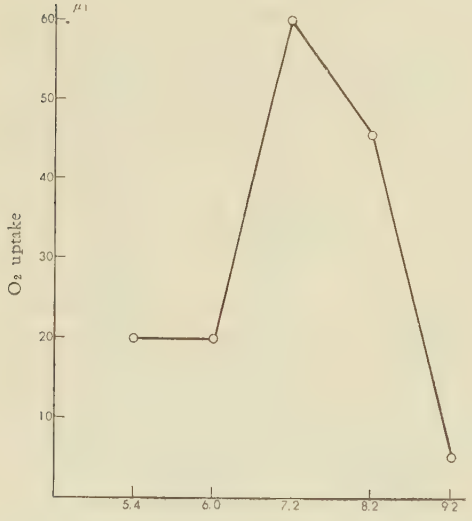


FIG. 3. 50°C Heat Treatment at several pH Range for 5 min. on Crude Extracts.
Substrates consentration....3 μM

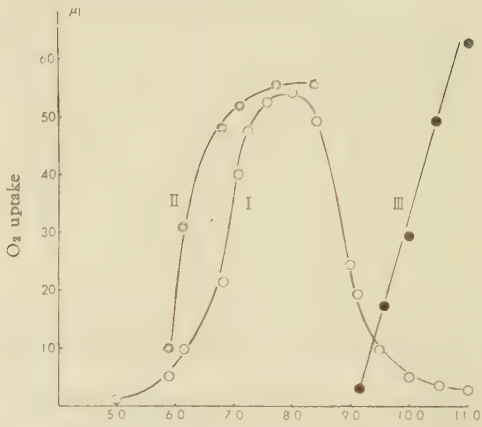


FIG. 4. The Effect of pH on Gentisic Acid Oxidase.
I: pH-activity curve, incubation of enzyme and substrate only
II: With ferrous ion 2×10^{-3} M and ascorbic acid
III: GA autooxidation
Phosphate buffer was use from pH 5.1 to 7.5 and Tris buffer from pH 8.0 to 9.0, and carbonate-bicarbonate buffer from pH 9.2 to 10.7.

TABLE VI. SUBSTRATE SPECIFICITY OF PARTIALLY PURIFIED SAMPLE

o-Hydroxybenzoic acid	0 ml
m-Hydroxybenzoic acid	0
p-Hydroxybenzoic acid	0
Gentisic acid	67.02
Protocatechuic acid	61.00
Homogentisic acid	0
Catechol	0
β-Resorcilic acid	0
2, 3-Dihydroxybenzoic acid	0

O_2 uptake in 30 minutes
Concentration of each substrates were 3 μM

as substrates. As shown in Table VI, none of the following compounds catechol, homogentisic acid, 2, 3-dihydroxybenzoic acid, and β-resorcilic acid were oxidized. Hence this enzyme differs distinctly from pyrocatechase and homogentisic acid oxidase. Though gentisic acid attacked as the substrate is out of question, it is a remarkable fact that protocatechuic acid is known to be attacked. From this phenomena, two possibilities are considered: a) the gentisic acid oxidase has also oxidative activity on protocatechuic acid, b) protocatechuic acid oxidase contaminates in this preparation. According to other experiments as will be described in the following paper[8], the cell free extracts from non-induced cells also attack PCA. Thus, it is clear that protocate-

chuic acid oxidase contaminates in this preparation and this strain has protocatechuic acid oxidase as the constitutive enzyme. The present authors have succeeded to separate both enzyme each other.

DISCUSSION

Gentisic acid oxidase is a new oxidase and requires ferrous ion as its sole co-factor similar to the other benzene ring splitting enzymes. It seems to be very interesting that these benzene ring splitting enzymes require ferrous ion, though a small difference exists in the liberation of ferrous ion from the apoprotein. In the case of gentisic acid oxidase, ferrous ion is libe-ated very easily by dialysis and salting out by ammonium sulfate. Futhermore since the use of phosphate buffer in extracting the enzyme from disrupted cells is unfavourable, because the precipitated, ferrous ion and phosphate ion complex, occurred and pH value decreased to some degree, we examined the application of trishydroxyaminomethane-HCl buffer for the purpose of extraction.

In order to obtain the crude extracts, disruption of the cells by application of ultrasonic vibration seemed to be a convenient method for large scale treatment. This enzyme, however, was labile by heat treatment and required ferrous ion as a co-factor which was easily oxidized, so that the extracts obtained by sonic disintegration lost their activity considerably. Therefore, the extracts were obtained by grinding with levigated alumina, with caution on cooling treatment and maintenance of reductive state during treatment.

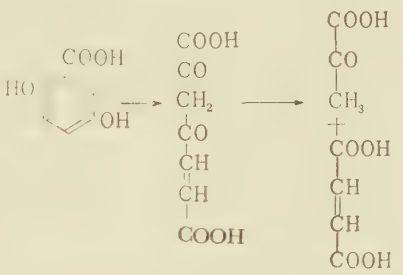
It should perhaps be possible to obtain the extracts without a loss of the activity caused by sonic treatment, paying attention to keep the content cooled and giving heed to the reductive agent.

SUMMARY

(1) Cell-free enzyme preparation which catalyzes an oxidative breakdown of gentisic acid with rupturing of benzene ring was obtained from *m*-hydroxybenzoic acid induced cells of *Pseudomonas ovalis* S-5. This new benzene ring splitting enzyme was named "Gentisic acid oxidase".

(2) Comparisons of various methods to obtain a cell free enzyme preparation were made. The enzyme was partially purified, and ferrous ion was proved necessary, for the activity of this enzyme. Further, some other properties of the gentisic acid oxidase were also investigated.

(3) From the various evidences obtained by manometrical experiments and chemical reaction of the product of this enzyme, the oxidative pathway of gentisic acid by *Ps. ovalis* was presumed to be as follows.



Acknowledgement The authors wish to express their sincere thanks to Honorary Prof. K. Sakaguchi for his continuous guidance throughout this work.

Metabolism of Aromatic Compounds by Microbes*

Part VIII. Further Studies of Gentisic Acid Oxidase

By Shin-ichi Sugiyama, Keiji Yano and Kei Arima

A highly purified gentisic acid oxidase from *Pseudomonas ovalis* S-5 was obtained and purification procedures are described in this report. The specific activity increased up to twenty fold. This enzyme specifically oxidizes gentisic acid with a consumption of 1 M of oxygen per 1 M of the substrate yielding fumarylpyruvic acid. Properties of this enzyme were extensively investigated. This enzyme required ferrous ion as sole cofactor. Several metal chelating inhibitors, analogue inhibitors and the reactivators as ascorbic acid, effect of preincubation and possible participation of SH-radical in the enzyme-substrate combination etc. were studied. And comparison of all the known five benzene ring splitting enzymes were made.

In the previons report[1], the authors obtained cell-free enzyme preparation from *Pseudomonas ovalis* S-5 which catalyzed the oxidative breakdown of gentisic acid (GA), and named it "gentisic acid oxidase." This enzyme, which required ferrous ion as a sole cofactor, was a new benzene ring splitting enzyme. The characteristics of gentisic acid oxidase show the similarity to the other enzymes as pyrocatechase, homogentisic acid oxidase.

The partially purified preparation consumed 1 M of oxygen per 1 M of GA and the carbon dioxide ovolution was not found. In the reaction mixture, an unknown substance which was colored specifically by *o*-phenylenediamine was accumulated. The authors presumed that this substance was the initial intermediate after the oxidative ring cleavage of GA, which might be maleylpyruvate or fumarylpyruvate.

As described in the later paper, we have recently succeeded in isolating one of the presumed intermediate in crystalline form[2]. Present communication is concerned with further purification and properties of this enzyme.

EXPERIMENTAL AND RESULTS

The biological source, the cultural conditions, the methods of induction of the enzyme, the method of extraction of the cell-free enzyme, and the method of determination of the enzyme activity were the same as described in the previous report. The determination of protein war conducted by Folin-Ciocalteu methods[3] after precipitation by 10 per cent trichloroacetic acid. In the previous report, M/15 phosphate buffer pH 7.2 was used for extraction of enzyme. However, when ferrous sulfate and ascorbic acid were added to enzyme preparation in order to maintain the level of cofactor and protecter, ferrous phosphate precipitated and pH value was lowered consequently. Hence Tris buffer (1/20 M trishydroxymethylaminomethane-HCl buffer pH 7.8) was newly prepared.

Further purification procedure.

As reported in the previous report, calcium-phosphate gel treated enzyme preparation, Fraction V still possessed oxidative activity on protocatechuic acid (PCA). Moreover, it, was found that the cell-free extracts of non-induced cells, which were obtained from culture using glucose as a sole carbon source, were also exhibited oxidative activity on protocatechuic acid[4]. So the

*Reprinted by permission of Agr. Chem. Soc. Japan from Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 3.

1) S. Sugiyama, K. Yano, K. Komagata and K. Arima, This Bulletin, **24**, 243 (1960).

2) S. Sugiyama, K. Yano, M. Tanaka and K. Arima, This Bulletin, **24**, 255 (1960).

3) O. Folin and V. Ciocalteu, *J. Biol. Chem.*, **73**, 729 (1927)

4) K. Yano and K. Arima, This Bulletin, in press.

authors concluded that this fraction contained at least two kinds of enzyme: protocatechuic acid oxidase and gentisic acid oxidase, and the former was constitutive enzyme in *Pseudomonas ovalis* S-5, and the latter was induced by the one of the inducer i.e, *m*-hydroxybenzoic acid (*m*-HBA). It was, therefore, desirable to separate the protocatechuic acid oxidase from gentisic acid oxidase as the first step in the further purification. The crude extracts were filtrated with 0.5 per cent hyflosupercel, and 0.2 volume of 10 per cent streptomycin sulfate was added with vigorous agitation to remove as much nucleic acid in crude extracts as possible. The precipitate resulted by low-speed centrifugation were discarded. The supernatant liquid was fractionated with neutral saturated ammonium sulfate and precipitate at 0.5 saturation was collected, and redissolved in 1/15 M pH 7.4 phosphate buffer with 10^{-3} M ferrous sulfate and ascorbic acid which were added as protectors against enzyme inactivation, and were dialyzed for 2 hours against the same buffer.

It was then adjusted to pH 7.4 sharply and protamine sulfate (20 mg per ml pH 7.0) was added slowly. The precipitate was removed periodically by centrifugation, and ratio of optical densities ξ 280 mμ/ξ 260 mμ was determined on a suitable dilution of the supernatant with Beckman spectrophotometer. The addition of protamine continued until this ratio reached at least 0.78, although crude extracts had a ξ 280 mμ/ξ 260 mμ ratio of about 0.65. After the protamine treatment the supernatant was treated with calcium phosphate gel. One-tenth volume of the gel was sufficient for a solution of the enzyme containing 7.5 mg of protein per mililiter. The mixture was immediately centrifuged an the supernatant liquid was collected.

As up to this step of purification on procedures, protocetechuic acid oxidase was not separated from the preparation, in order to separate protocatechuic acid oxidase from gentisic acid oxidase, it was then refractionated by ammonium sulfate. The enzyme protein which precipitates between 0.15 and 0.33 saturation was collected, and redissolved in dist. water. This was then adjusted to pH 7.4 with aqueous ammonia, and this preparation was dialyzed for 1 hour against dist. water at pH 7.0, containing 10^{-3} M Fe^{++} and ascorbic acid. It was then retreated with 1.25 volume of calcium phosphate gel, and the mixture was adjusted sharply to pH 7.4 by addition of 1 N acetic acid. After fifteen minutes standing the mixture was centrifuged, then the collected gel was carefully washed twice, with dist. water and with M/150 phosphate buffer, and eluted with one-tenth of the initial volume of pH 7.6 M/15 phosphate buffer solution. The elution was repeated again, and clear transparent straw colored enzyme solution was obtained. The activity of this enxyme solution was calculated to have the Q_{O_2} value (protein) of 8000. Table I shows the specific activity and the yield of the enzymes. As seen in this Table, the yield of the enzyme was about 10 per cent of the original broth in spite of careful treatment. The amount of purified material available was insufficient for further purification. These loss of yield could not be recorved by addition of SH-protector as glutathione, cystein and ascorbic acid to Fraction VII.

Separation of the two enzymes.

The enzyme fraction between 0.15 and 0.33 saturation of ammonium sulfate oxidized GA and the supernatant liquid oxidized PCA respectively. In our experiment, separated protocatechuic acid oxidase also required ferrous ion as cafactor.

Protocatechuic acid oxidase was already studied by Stanier et al.[5] in detail. They have demonstrated that ferrous ions were not required by protocatechuic acid

5) R. Y. Stranier and J. L. Ingraham, *J. Biol. Chem.*, **210**, 799 (1954).

Table I. Purification of Gentisic Acid Oxidase

Fraction		Volume	Protein		Specific activity	Yield
			mg/ml	total	Q_{O_2} (protein)	per cent
Crude extracts	(I)	200 ml	13.6 mg	2,720 mg	413	100
Treated with Hyflosupercel	(II)	176	12.7	2,366	439	92.5
Treated with Streptomycin	(III)	240	8.8	1,848		
ppt. by $(NH_4)_2SO_4$ 0.5 satu.	(IV)	40	11.4	456	952	38.7
After Ca-Ph. gel negative absorp.	(V)	40	6.0	272	1,412	34.2
After refraction by $(NH_4)_2SO_4$ 0.15~0.33 satu.	(VI)	20	3.7	72	3,789	24.4
After Ca-Ph. gel positive absorp.	(VII)	9	1.7	15.5	8,002	10.9

oxidase preparation which was extracted from *Pseudomonas fluorescens* A-3-12. This difference may be caused due to the difficulty of the preparation of iron-free enzyme, and does not preclude the possibility that iron is a component of both preparation. In the case of *Ps. fluorescens*, ferrous ions might be released from enzyme protein by the more strict treatmeut.

Specificity

The oxidation of several aromatic compounds by this enzyme fraction were tested. As seen in Table II the only compound proved to be attacked was GA. Fumarylpyruvic acid which was the intermediate after eleavage of GA[2] was also tested but showed negative result.

TABLE II. SUBSTRATE SPECIFICITY OF GENTISIC ACID OXIDASE

Substrate	Oxygen uptake
Gentisic acid	67.0 μl
Homogentisic acid	0.0
Protocatechuic acid	0.0
2, 3-Dihydroxy benzoic acid	0.0
α-Resorcilic acid	0.0
β-Resorcilic acid	0.0
o-Hydroxy benzoic acid	0.0
m-Hydroxy benzoic acid	0.0
p-Hydroxy benzoic acid	0.0
Phenylacetic acid	0.0
Catechol	0.0
Fumaric acid	0.0
Pyruvic acid	0.0

Each substrates were added as sodium salt.
Concentration of each substrates 3 μM, and activity was indicated by the oxygen uptake for 5 min.

The preservation of enzyme with ferrous ions.

The ferrous ion free enzyme solution was adjusted at pH 7.2 with 1 N HCl and incubated in a water bath regulated at 30°C. On the other hand, 10^{-3} M ferrous ions was added to this preparation and incubated in the same condition. Both samples, incubated for 0, 10, 20, 30, 45, and 60 minutes, were cooled immediately to room temperature and readjusted to pH 8.0 with 1 N NH_4OH. Then activities of these enzyme preparations were determined by use of Warburgs manometer. In the determination, only ascorbic acid was added in the ferrous-enzyme preparation, whereas to the iron-free preparation both ascorbic acid and ferrous ions were added. As seen in Table III, activity of ferrous ion-enzyme preparation didn't remarkably decreased, but loss of the activity of iron-free enzyme occurred with the elapse of time, and anaerobic condition could not prevent the loss of activity.

The combination of iron and enzyme.

Fig. 1 shows the effect of a several combinations of

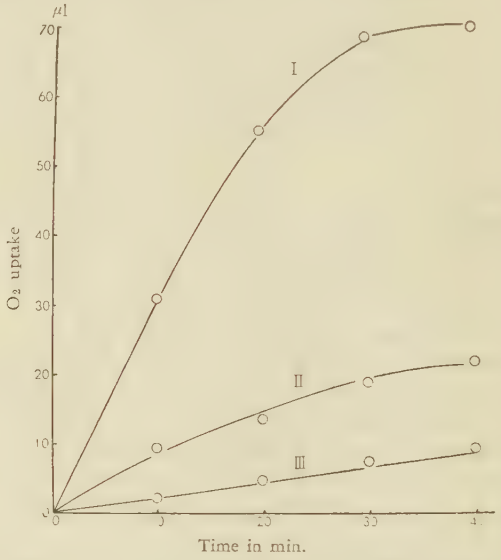


FIG. 1. Activity of Iron Free Enzyme Preparation preincubated with Ferrous Ions.

Curve I: Preincubated ion-free enzyme with ferrous ion
II: Preincubated ion-free enzyme with substrate
III: Preincubated ferrous ion with substrate
The reaction was initiated by mixing three components after the preincubation.
Ferrous ion; final 10^{-3} M with ascorbic acid.

TABLE III. THE EFFECTS OF INCUBATION TIME WITH FERROUS ION AT 37°C, pH 7.2

	time	0	10	20	30	45	60	min
I.	Fe^{++} added	100	100	100	98	95	92	activity
II.	not-added	100	82	70	61	52	38	per cent

I; Ferrous Ion 10^{-3}M was added.
Enzyme activity was determined by manometry after incubation.
And in the determination, ascorbic acid 2×10^{-3}M was added to I, and ascorbic acid 2×10^{-3}M with ferrous ion 10^{-3}M to II.

iron free enzyme preparation and ferrous ions. Iron free enzyme was prepared by dialysis of purified enzyme. Three systems were tested; 1) preincubation of the mixture of ferrous ions, ascorbic acid and the enzyme, 11) enzyme and substrate, and 111) ferrous ions, ascorbic acid and substrate respectively. The preincubation was carried out in Warburgs vessels before enzymation. As shown in Fig. 1, when the substrate was added to a preincubated system of ferrous ions, ascorbic acid and enzyme 1), the activities were greater than those of 11) or 111). It was suggested that the formation of a metal-protein complex occurred in the case of 1), and this complex was necessary for the activity of this enzyme. The effects of preincubation were not reported on other benzene ring splitting enzymes.

In the case of aconitase, which required ferrous ion as cofactor as like benzene ring splitting enzymes, it was founded that the ferrous ion-enzyme complex acted as substrates, and this enzyme showed greater activity in the presence of this chelate than the substrate alone.

Action of inhibitors

Table IV shows the effect of various inhibitors on the enzyme. This enzyme was extremely inhibited by several metal chelators such as α, α'-bipyridyl, *o*-phenanthroline, hydroxyquinoline and nitroso-R-salt, and also by metal ions and KCN. Inhibition per cent of KCN markedly differed depending on the concentration of CN^-, which might be due to the production of cianhydrine.

The inhibition of chelators was recovered by the addition of ferrous ion with ascorbic acid 10^{-3} M, but not by the addition of ascorbic acid or other reducing agents alone. Table V shows the effects of chelating reagents and recovery of activity by ferrous ion.

TABLE IV. THE EFFECT OF SEVERAL INHIBITORS

Inhibitor	concentration	inhibition per cent
Monoiodoacetate	1×10^{-3} M	11.2
p-Chloromercury-benzoate	1×10^{-3}	12.6
NaF	1×10^{-2}	4.0
Semicarbazide	1×10^{-2}	17.6
$AgNO_3$	1×10^{-3}	100.0
	1×10^{-4}	53.9
$CuSO_4$	1×10^{-3}	60.5
	5×10^{-4}	57.3
α, α'-Bipyridyl	1×10^{-3}	80.0
Ethylenediamine-tetraacetic acid	1×10^{-3}	74.0
8-Hydroxyquinoline	1×10^{-3}	110.0
o-Phenanthroline	1×10^{-3}	100.0
KCN	1×10^{-3}	100.0
	1×10^{-4}	39.5
NaN_3	1×10^{-3}	29.5

TABLE V. THE EFFECT OF CHELATING REAGENTS AND RECOVERY OF ACTIVITY BY FERROUS ION

Chelating reagent	Inhibition per cent	$Fe^{++}(10^{-3}M)$ added recovery per cent
none		100.0
α, α'-Bipyridyl	50.0	98.0
o-Phenanthroline	38.0	80.0
8-Hydroxyquinoline	46.0	93.0
Nitroso *R* salt	21.0	75.0

TABLE VI. INHIBITION BY *p*-CHLOROMERCURY-BENZOATE

PCMB Concentration	I E+S+(PCMB)	II (E+PCMB)+S	III (E+S)+PCMB
10^{-4} M	12.0	40.8	7.2
10^{-3} M	46.1	82.4	21.5

Values are shown by inhibition per cent.
E; enzyme. S; substrate, PCMB; *p*-chloromecury-benzoate.
I; PCMB was adeed at the start of reaction.
II; PCMB was preincubated with enzyme for 30 min. before reaction.
III; PCMB was added 7 min. after the start of reaction.

The effect of SH-reagent.

p-Chloromercury benzoate and monoiodoacetic acid in the concentration at 10^{-3} M showed almost no inhibition when they were added at the start of the enzyme reaction. When preincubated with enzyme, however, these substances inhibited enzyme reaction very clearly. These are specific SH-inhibitors, so the activity of this enzyme might be attributed to SH-radical. Table VI shows the activity of enzyme (E) preincubated with *p*-chloromercury-benzoate (PCMB) and with substrate (S) in various combinations. In each case reactions were carried out in the Warburgs vessel at 30°C. The inhibition in the combination (II) was higher than that in the case of (I), irrespective of the PCMB concentration. And in the case of (III), it was always lower than that of (I). It was suggested that the formation of enzyme-substrate complex was related to SH-radical in some manner. This inhibition was recovered to some degree by addition of glutathione, although only a few recovery was found in the case of the enzyme combined with PCMB for a long time.

Furthermore, it was proved that such purified preparation did not contain a spectrophotometrically detectable amount of flavin.

Analogue inhibitions.

Inhibition by the analogues of GA were examined.

TABLE VII. INHIBITION BY ANALOGUE COMPOUNDS OF GENTISIC ACID

Compounds in vessels	concentration	activity O_2 uptake in 30min.
Gentisic acid (G)	2.9×10^{-3} M	61.04
(G) and Homogentisic acid	2.9×10^{-3} 2.9×10^{-3}	7.27
(G) and Homogentisic acid	2.9×10^{-3} 2.9×10^{-3}	18.09
(G) and *p*-Hydroxybenzoic acid	2.9×10^{-3} 2.9×10^{-3}	59.8
(G) and *m*-Hydroxybenzoic acid	2.9×10^{-3} 2.9×10^{-3}	61.0
(G) and *o*-Hydroxybenzoic acid	2.9×10^{-3} 2.9×10^{-3}	62.5
(G) and 2, 3-Dihydroxybenzoic acid	2.9×10^{-3} 2.9×10^{-3}	61.0
(G) and Protocatechuic acid	2.9×10^{-3} 2.9×10^{-3}	60.04
(G) and α-Resorcilic acid	2.9×10^{-3} 2.9×10^{-3}	64.02
(G) add β-Resorcilic acid	2.9×10^{-3} 2.9×10^{-3}	62.02
(G) and Catechol	2.9×10^{-3} 2.9×10^{-3}	61.04
(G) and Resorcin	2.9×10^{-3} 2.9×10^{-3}	45.9
(G) and Hydroquinone	2.9×10^{-3} 2.9×10^{-3}	9 1

As seen in Table VII, none of these compounds including protocatechuic acid were oxidized, which indicates that the Fraction VI was highly specific for the oxidation of GA. The relative afinities of each of these analogues to the enzyme were tested by determining their individual effects on the rate of oxidation to an equimolar concentration of GA. Except hydroquinone (HQ), and homogentisic acid (HGA), which were inhibitory, none inhibited the initial rate of oxygen uptake significantly.

It was hery interesting that only HQ and HGA, in which hydroxy radicals occupies, the positions of 2 and 5 as GA, inhibited gentisic acid oxidase activity. The inhibitory actions of these compounds, therefore, may be attributable to the similar specific composition between GA and these compounds. The typical competition curve, however, has not been obtained in the present experiment.

DISCUSSION

We assumed that this enzyme was related to other oxidases which act upon phenolic compounds: homogentisic acid oxidase[6~10], pyrocatechase[11~13], protocatechuic acide oxidase[5], since in each case, molecular oxygen is an obligatory oxidant, and two atoms of oxygen are consumed in the reaction, the products are unsaturated di- or tri-carboxylic acid. These four enzymes bring about extremely specific cleavages of the benzene ring. Their known properties are summarized in Table VIII. R. Y. Stanier et al.[5] have reported that neither glutathione nor ferrous ions requirement could be shown at any stage in the purification of protocetechuic acid oxidase. More recently, however, S. Dagley[14] and E. L. Tatum[15] have reported that ferrous ion is required as cofactor for protocatechuic acid oxidase. It was assumed that this striking difference may be quantitaive and does not preclude the possibility that iron in a component of all these enzymes.

The optimum concentration of required ferrous ions for the purified enzyme preparation existed between 10^{-3} M and 10^{-4} M, when examined by Warburgs manometer. However, it is doubtful that whether iron is competely free from the enzyme preparation or not, so that the enzyme should be further purified, if ferrous ions concentration should be exactly determined.

Maleyl-puruvate, an initial intermediate of the ring fission was recognized as *cis*-form by spectrophotometrical assay of the reaction mixture after 15 minutes incubation using this enzyme. It is believed that GA is oxidized with benzene ring fission to *cis*-form aliphatic compound, maleylpyruvate, and then is transformed to the isomer of *trans*-form, fumarylypyruvate. W. E. Knox et al.[10] have reported that HGA was oxidized to fumarylacetoacetate by glutathione requiring isomerase obtained in cell-free extracts. Our spectrophotometrical experiments

6) M. Suda and Y. Takeda, *J. Biochem.*, **37**, 381 (1850).
7) R. G. Ravdin and D. J. Crandall, *J. Biol. Chem.*, **189**, 137 (1951).
8) D. I. Crandall and D. N. Halkis, *J. Biol. Chem.*, **208**, 629 (1954).
9) D. I. Crandall, *J. Biol. Chem.*, **212**, 565 (1955).
10) W. E. Knox and S. W. Edward, *J. Biol. Chem.*, **216**, 479 (1955).
11) O. Hayaishi and K. Hashimoto, *J. Biochem.*, **37**, 371 (1950).
12) O. Hayaishi and K. Hashimoto, *Med. J. of Osaka Univ.*, **2**, 33 (1950).
13) O. Hayaishi, M. Katagiri and S. Rothberg, *J. Biol. Chem.*, **227**, 889 (1957).
14) S. Dagley and M. D. Patel, *Biochem. J.*, **66**, 227 (1957).
15) L. Ottery and E. L. Tatum, *J. Biol. Chem.*, **223**, 307 (1956).

TABLE VIII. COMPARISON OF THE RING SPLITING ENZYMES

Enzyme	Inhibiton	Reveral of inhibition	Analogue inhibition	Opt. pH	Reference
Gentisic acid oxidase	Dialysis	Fe^{++}+ascorbate	Homogentisic acid	8.0	This paper
	α, α'-Bipyridyl	// //			
	Oxyquinoline	// //			
	o-Phenanhroline	// //	Hydroquinone		
	Nitroso *R* salt	// //			1.
	PCMB	Fe^{++}+GSH			
	KCN				
Homogentisic acid oxidase	Dialysis	Fe^{++}+ascorbate	Hydroquinone	7.0~7.2	6, 7, 8, 9, 10.
	H_2S, Cystein				
	KCN				
	α, α'-Bipyridyl	Fe^{++}+ascorbate			
	Versene	Fe^{++}			
	Oxine				
	o-Phenanthroline				
	PCMB	Fe^{++}+GSH			
Pyrocatechase	Dialysis	Fe^{++}		8 0	11, 12, 13.
	α, α'-Bipyridyl				
	o-Phenathroline				
	PCMB	GSH			
Protocatechuic acid oxidase	PCMB	GSH	Catechol	probably 9.0	5, 14, 15.
	Nitroso *R* salt	Fe^{++}+ascorbic	2, 3- and 2, 4-Dihydroxy-benzoic acid		4.
3-Hydroxy-anthranilic acid oxidase	α, α'-Bipyridyl				16.
	Oxine				
	Versene				
	Citric acid				

indicated that *cis*-form intermediate was isomerized spontaneously to the *trans*-form. However, when dialyzed crude enzyme reacted on GA, it only produced the *trans*-form intermediate, so that *cis-trans* isomerization must have been carried out by an isomerase existed in the crude extracts.

SUMMARY

1. A highly purified gentisic acid oxidase preparation free from protocatechuic acid oxidase was obtained by the repeated ammonium sulfate precipitations. The specific activity increased up to twenty-fold. This enzyme preparation is free from oxidases of other aromatic compounds, as maleylpyruvate isomerase and fumarylpyruvate hydrolyase.

2. The activity of this enzyme preparation was remarkably inhibited by metal reagents such as α,α'-dipyridyl, ethylenediaminetetraacetic acid, *o*-phenanthroline and nitroso-R-salt, and the inhibition was reversed by the addition of ferrous ions, and reactivation increased by the further addition of reducing agents, but not by the reducing agent such as ascorbic acid alone.

3. Enzyme and ferrous ion combination was proved to the necessary for the activity of the gentisic acid oxidase as the results of experiments using several combinations of iron-free enzyme, substrate and ferrous ions.

4. The loss of activity of iron-free enzyme could be recovered by the addition of ferrous ion. Other metalic ions and reducing agents tested were not effective.

5. Participation of SH-radical in the enzyme-substrate combination was presumed from the inhibition pattern by SH-reagents such as *p*-chloromercury-benzoate.

The authors wish to express their sincere thanks to Honorary Prof. K. Sakaguchi for his continued guidance throughout this research.

16) M. H. Mehler, *J. Biol. Chem.*, **218**, 241 (1956).

Metabolism of Aromatic Compounds by Microbes*

Part IX. The Enzymatic Conversion of Gentisic Acid to Fumarylpyruvic Acid

By Shin-ichi SUGIYAMA, Kirosato TANAKA, Keiji YANO and Kei ARIMA

One of the presumed product after the ring cleavage of gentisic acid by the gentisic acid oxidase free of hydrolysing enzyme from *Pseudomonas ovalis* S-5 was obtained in a crystalline form. This new substance was proved to be a β-diketodicarboxylic acid and was identified as fumarylpyruvic acid, as a results of investigation of the chemical and physical properties. Furthermore, maleylpyruvic acid, the initial product of the ring cleavage of gentisic acid oxidation was recognized in the reaction mixture using the purified enzyme. This is proved to be transformed to fumarylpyruvic acid, which then was hydrolyzed into pyruvic acid and fumaric acid by crude cell free extract. Thus the pathway of gentisic acid oxidative metabolism was established.

The authors had already investigated the metabolism of *m*-hydroxybenzoic acid by *Pseudomonas ovalis* S-5[1]. This acid was oxidized to gentisic acid and then the oxidative ring cleavage of gentisic acid was ocurred[2]. The enzyme which catalized the reaction of the gentisic acid oxidation named gentisic acid oxidase was obtained[2] as the cell-free preparation and its purification, its properties were studied and described in the previous reports[3] in which the authors presumed from several experimental results that ring cleavage of gentisic acid may occur between carbons 1 and 2 giving rise a β-kideto-dicarboxylic acid: fumarylpyruvic acid or maleylpyruvic acid (Fig. 1).

Recently we have succeeded in isolating one of the direct ring cleavage product in a crystalline form. From the results obtained the crystalline intermediate was identified as fumarylpyruvic acid. The presence of a *cis* intermediate maleylpyruvic acid was also confirmed spectrophotometrically in the reaction mixture using purified enzyme preparation.

*Reprinted by permission of Agr. Chem. Soc. Japan from Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 3.

1) S. Sugiyama, K. Yano, K. Komagata, M. Kazama and K. Arima, This Bulletin, **24**, 211 (1960).

2) S. Sugiyama, K. Yano, K. Komagata and K. Arima, This Bulletin, **24**, 243 (1960).

3) S. Sugiyama, K. Yano and K. Arima, This Bulletin, **24**, 249 (1960).

EXPERIMENTS AND RESULTS

Enzyme preparation *Pseudomonas ovalis* S-5 was grown in bouillon peptone medium for 18 hrs. with constant shaking, harvested after this by centrifugation and washed with dist. water. The resting cells thus obtained were induced by *m*-hydroxybenzoic acid for three hrs. in Sakaguchi flasks at 30°C[4].

The induced cells harvested by centrifugation were ground by hand with the same weight of alumina until breakage occured.

The mixture was suspended in 2 ml of 0.05 M Tris-hydroxyaminomethane-HCl buffer (pH 7.8) per gm. of wet cells and the bulk of the alumina was removed by low speed centrifugation. The extract was centrifuged at 10,000×g for 30 minutes to remove unbroken cells. All procedure was carried out in the cold room (2°C±2°). For the purpose of obtaining the ring cleavage product it was only necessary to remove the hydrolysing enzyme. The accumulation of the intermediate in the reaction mixtures using non-dialyzed extracts for 20 hrs., 40 hrs., and more than 40 hrs. were carried out, and the dialyzed crude extracts were investigated with *o*-phenylenediamine reagent. *o*-Phenylene-

4) K. Arima, K. Komagata, S. Sugiyama, K. Kazama and K. Yano, This Bulletin, **19**, 51 (1954).

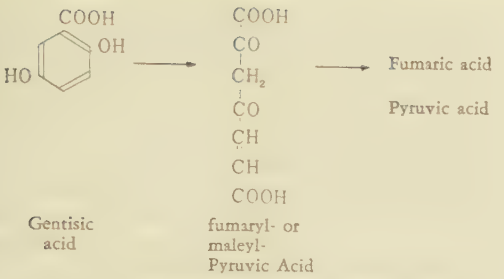


FIG. 1. Pathway of Oxidative Metabolism of Gentisic Acid

diamine reagent were prepared according to the method described by R. F. Witter et al.[5]. The reactions were carried out for 30 min. in Warburg's vessel containing 0.5 ml enzyme preparation, 0.5 ml M/10 tris buffer pH 7.8, 0.3 ml M/10 sodium gentisate, and 0.1 ml M/100 ferrous sulfate and ascorbic acid mixture. Half volume of trichloroacetic acid was added to the reaction mixture and then centrifuged at 3,000 r.p.m. for 15 minutes. 0.25 ml of the supernatant was diluted to 6 ml with dist. water and 1 ml of *o*-phenylenediamine regents was added. The these were allowed to stand for 30 min. at room thmperature. The optical densities of reaction mixtures with *o*-phenylenediamine were measured by HITACHI spectrophotometer model 40 P.

The more the crude enzyme extracts dialyzed, the more the optical density of reaction mixture with *o*-phenylenediamine increased. However, no increase was observed after 40 hrs. dialysis. So the enzyme preparation for the accumulation of the intermediate could be obtained by dialyzing the crude extracts for 50 hrs. against dist. water which was adjusted to pH 8.0 with aqueous ammonia.

Isolation and purification of the intermediate.

The dialyzed crude enzyme extracts prepared as described above was diluted 8 folds with M/10 Tris-aminomethane-HCl buffer pH 8.0.

The enzymatic oxidation was carried out in 500 ml Sakaguchi flask containing 50 ml of M/10 sodium gentisate, 5 ml of 10^{-2} M aqueous solution of ferrous sulfate and ascorbic acid, and a small quantity of sodium bicarbonate, with constant shaking for 30 to 40 minutes. The pH of the reaction mixture without sodium bicarbonate would go down as low as 5 during the incubation and the oxidation reaction would stop accordingly, leaving not a small quantity of sodium gentisate unoxidized. After 40 minutes incubation, no sodium gentisate was detected in the reaction mixture by Folin's phenol reagent.

The reaction mixture was deproteinized with 0.1 volume of 20 per cent metaphosphoric acid and centrifuged. The supernatant was extracted continuously with peroxide free ethyl-ether for about 50 hrs. The ether was removed from the extract under reduced pressure in a desiccator. Then yellow crude intermediate was obtained. The yellow crude intermediate was dissolved in ethylacetate and then petrolium ether was added until slight precipitation was found. This was allowed to stand at −20°C over a night. Slightly yellow precipitate thus obtained was made free of ethylacetate and petroleum ether under reduced pressure and then dissolved in a small amount of ethanol. More than 10 times benzene to ethanol in volume was added to the almost saturated ethanol solution. Then it was stood under reduced pressure with special precaution. It was discontinued just before the solution became turbid. The crystallization did not occur by merely adding benzene dropwise to the saturated ethanol solution. The crystalline intermediate appeared after standing it at 4∼5°C over a night of two. It was recrystallized three times from ethanol-benzene mixture as described above.

Properties of the intermediate.

Elementary analysis An analytical sample was prepared by careful drying of the pure intermediate with an Abderhalded aparatus.

Anal. Calcd. for $C_7H_6O_6$; C 45.17 ; H 3.25
Found C 45.20 ; H 3.40
dec. P. 180∼181°C

This intermediate gave a sharp yellow color reaction with *o*-phenylenediamine. It also readily reacted with carbonyl reagents such as sodium nitroprusside, 2, 4-dinitrophenylhydrazine and semicarbazide.

Titration of the intermediate. Direct evidence for the dicarboxylic and enolic nature was obtained by titrating with alkali with use of glass electrode. 45 mg of the intermediate was dissolved in 3 ml of ethanol and 1 ml of water and titrated with M/10 sodiumhydroxide. The titration curve (Fig. 2) showed that there were two carboxyl groups ; their pK values appear in this condition to be at 3 and 4.4 respectively.

Further titration up to pH 12 revealed the presence of an additional ionizable group with pK at 7.8 (presumably representing the enolic form of the β-diketo group).

UV spectra of the crystalline intermediate. As shown in Fig. 3, this compound had a strong absorption

5) R. F. Witter and E. Stotz, *J. Biol. Chem.*, **176**, 501 (1948).

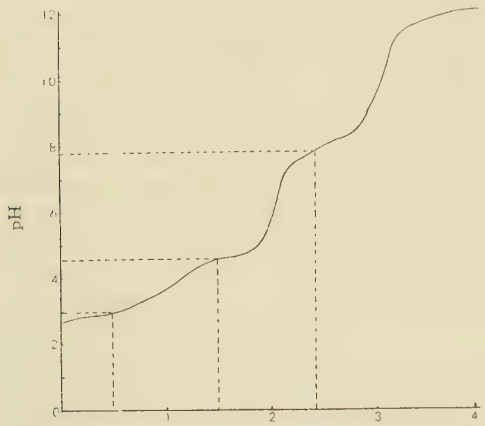


FIG. 2. Titration Curve of the Intermedaite.

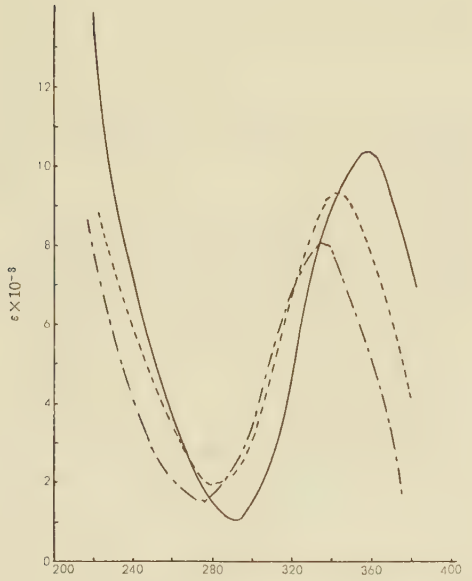


FIG. 3. Ultraviolet Absorption Spectra of the Intermediate.

near 340 mμ which slightly shifted when pH was raised. The maximum molar extinction coefficients for this compound were 8,300 at 335 mμ in 0.1 N HCl; 9,400 at 340 mμ in phosphate buffer at pH 7.3; 10,300 at 350 mμ in 0.1 N NaOH. These facts were also compatible with the presumed structure of fumarylpyruvic acid. Judging from those molar extinction coefficient values, we should presume that most of this acid exists in *mono*-enol form even in acidic side.

Infra-red spectrum of the intermediate. Infra-red spectrum of this compound in Nujol mull is shown in Fig. 4. A deep absorption band at 1,710 cm^{-1} was considered to be due to C=O of unsaturated carboxyl radical. A characteristic broad band in the range 1,630~1,500 cm^{-1} was attributed to the conjugated chelation of *mono*-enol β-diketone structure.

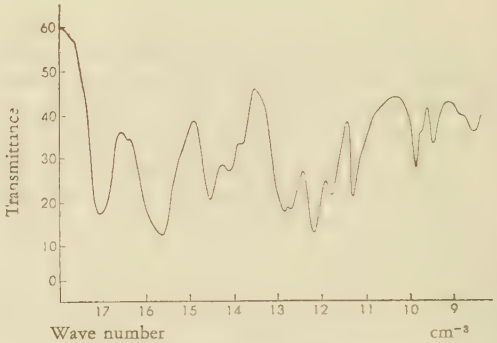


FIG. 4. The Infra-red Spectrum of the Intermediate.

The sharp band at 980 cm^{-1} was assigned to the out-of-plain deformation vibration of the hydrogen atoms of *trans*-ethylenic linkage.

Enzymatic hydrolysis of the intermediate. A further fact to confirm the structure of fumarylpyruvic acid and its metabolic fate in this *Pseudomonas* strain was obtained by hydrolyzing the intermediate by the crude enzyme extracts prepared from the *m*-HBA induced cells.

As described above, the crude extract of *m*-HBA induced cells which had not yet dialyzed contained the fumarylpyruvic acid hydrolyase which hydrolyzes fumarylpyruvic acid.

The crude extracts were free of the particulate fraction by centrifugation at 20,000×g. So the crude extract was thought to be free of the cyclophorase. This fact was also confirmed by estimating the oxidizability of this extract toward fumaric acid, succinic acid, and maleic acid. None of these compounds were oxidized.

5 mg of pure intermediate was dissolved in M/10 Tris-aminomethane-HCl buffer at pH 7.8 and added to 5 ml of non-dialyzed crude extracts to final volume of 10 ml, and was incubated at 30°C for 1.5 hrs. After the incubation, no *o*-phenylenediamine positive substance was

detected in the reaction mixture. The reaction mixture was deproteinized with metaphosphoric acid and then adjusted at pH 2.0 with 1 N H_2SO_4 and continuously extracted with peroxide free ether.

The acids in the extract were identified chromatographically on the paper sprayed with 0.2 per cent bromophenol blue, using the upper layer of butanol 4 : acetic acid 1 : water 3, as its developing solvent. The extracts yielded two distinct acidic spots which showed the same R_F values with fumaric acid and pyruvic acid respectively.

The same procedures were carried out with 30 μM of GA as the substrate, and without substrate. When gentisic acid was used as the substrate, the result obtained by paparchromatography was the same as in the of the fumarylpyruvic acid was used as the substrate. No spot was detected by spraying B.P.B. (bromopheenol blue) solution except two, fumaric acid and pyruvic acid (Fig. 5). When the crude extracts were incubated without substrate, no acidic spot was detected at all. These facts showed that GA was metabolized via the intermediate into fumaric and pyruvic acids, and the crystalline intermediate is 7 carbon fumarylpyruvic acid. Physical and chemical properties of this new substance,

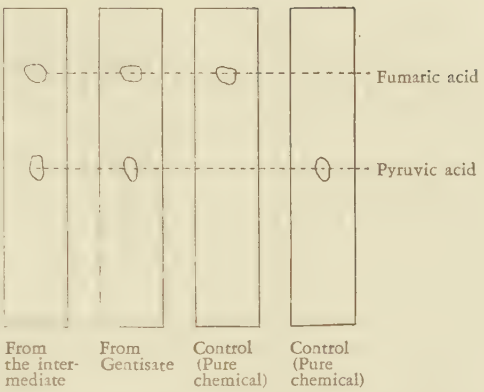


FIG. 5. Paperchromatogram of the Enzymatic Hydrolysates of the Intermediate.

fumarylpyruvic acid were summerized in Table I.

Dection of *cis* form intermediate, meleylpyruvic acid.

To know the existence of the *cis* form intermediate, the authors employed the purified preparation of gentisic acid oxidase, described in the previous report, which was free of the isomerase converting *cis* intermediate into *trans*

TABLE I. PROPERTIES OF THE NEW SUBSTANCE, FUMARYLPYRUVIC ACID

Formula	$C_7H_6O_6$ *anal.* calcd. C : 45.17, H : 3.25, O : 51.28 found. C : 45.20, H : 3.40	
Molecular weight	186.12	
Crystalline form	Bipyramid (in ethanol-benzene)	
Color	Slightly yellow	
m.p. (dec.)	180～181°C	
pK	$pK_1=3.0$, $pK_2=4.4$, $pK_3=7.8$ (in ethanol 3 : water 1)	
Positive reagents	2, 4-Dinitrophenylhydrazine Semicarbazide Sodium-nitroprusside *o*-Phenylenediamine	
Ultraviolet absorption	pH 1 ; 335 mμ (ε=8,300), pH 7.5 ; 340 mμ (ε=9,400), pH 13 ; 350 mμ (ε=10,300),	
Infrared absorption	C=O Vibration, α-β-unsaturated acid	1,710 cm^{-1}
	C—O– Stretching vibration,	1440～1935
	C=C Stretching vibration	1320～1211
	C=C Conjugated	near 1600
	C—H Out of plane defermation vibration (–CH=CH– *trans*)	980
	R–C=CR′–––C–R″ Conjugated chelation, monoenol-diketone O – O H	1560～1570

form fumarylpyruvic acid.

The enzymatic oxidation was carried out in a Warburg's vessel containing 3 μM of GA, 0.25 ml of the purified enzyme solution, 0.75 ml of Tris buffer with 0.1ml of ferrous sulfate and ascorbic acid solution (10^{-3}M) at 30°C for 15 min. The reaction mixture was deproteinized immediately with 0.5 ml of 10 per cent trichloroacetic acid, centrifuged, and the precipitate was discarded. And the supernatant was neutralized with sodium hydroxide solution. The absorption spectra of this preparation is shown in Fig. 6. The reaction mixture absorbed strongly near 340 mμ at pH 13 and it showed but a very weak absorption at pH 1.5. The absorption in acid solution at 340 mμ increased when it was allowed to stand at pH 1.5 at room temperature (see Fig. 6). These results might be due to the fact that the first degradation product of GA oxidation was maleylpyruvic acid which absorbed strongly only in alkaline solution and then the maleylpyruvic acid was converted into the *trans* intermediate, fumarylpyruvic acid, which absorbed strongly both in alkaline and acid solutions. This conversion was conducted probably by an enzyme in crude extracts, but not by merely standing in an acid solution at room temperature.

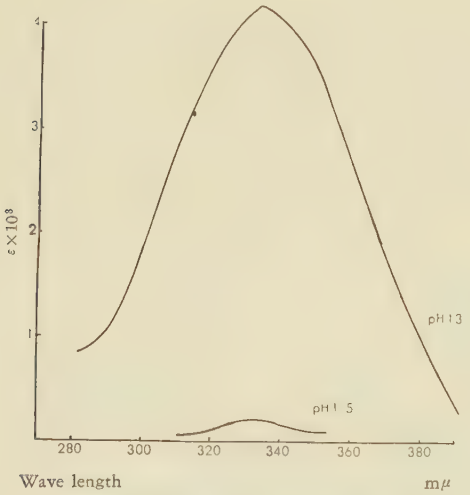


FIG. 6. Ultra Violet Absorption of the *cis* Isomer Maleylpyruvic Acid.

Determination of fumarylpyruvic acid.

Fumarylpyruvic acid was determined principally by the method of R. F. Witter et E. Stotz[5] which was the colorimetric determination of β-diketone using *o*-phenylenediamine. Since values by their method were not always linear function, a modification of it was carried out.

An amount of 100 mg *o*-phenylenediamine is dissolved in 25 ml phosphate-sulfric acid reagent, freshly prepared daily. A sample which contained about 20~100 μg of fumarylpyruvic acid was filled up to 10 ml with dist. water and added 2 ml of *o*-phenylenediameine regent, allowed to stand at room temperature for 30 min. The aquous solution was transported to 30 ml volume separating funnel and shaked with 10 ml ethylether. The substance which was produced by the reaction between fumarylpyruvic acid and *o*-phenylenediamine moved into upper ether layer, but excess *o*-phenylenediamine remained in aquous layer, which was discarded. 10 ml of 14 per cent Na_2CO_3 aquous solution was added to ether solution and shaked in the separating funnel. Yellow substance, then moved to aquous layer. And this color was measured by spectophotometrically at 440 mμ. Determination was carried out with water as control. Relation between optical density and concentration of fumarylpyruvic acid is shown in Fig. 7. Pyruvic acid gives no infulence to this determination.

FIG. 7. Relation between optical Density and Concentration of Fumarylpyruvic acid.
Condition of determination is discribed in this papar.

DISCUSSION

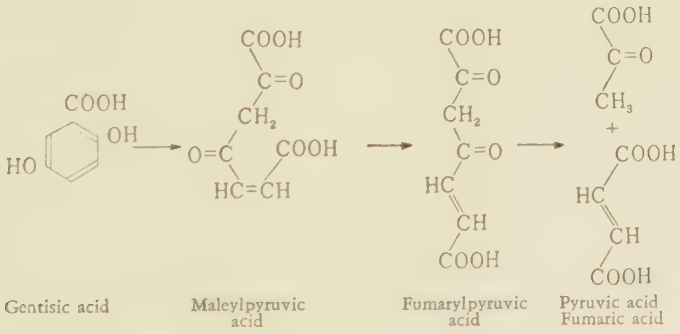
The ultraviolet absorption spectrum of β-diketone shows the maximal spectrum near 330 mμ in alkaline solution. A. Meister reported that characteristic absorptions of triacetic acid and other β-diketones are enhanced by alkali, and suppressed by acid[6,7].

Fumarylpyruvic acid which absorbes strongly both in acid and alkaline solutions has a similar spectra reported for fumarylacetoacetic acid by R. G. Ravdin et al.[8], who reported that the striking pH stability of the spectrum appeared to be a reasonable property for fumarylacetoacetic acid on theoretical grounds. The fumaryl double bond of fumarylacetoacetic acid forms as conjugated system with either the keto or enol form of the δ-carbonyl group. Succinylacetoacetic acid which is saturated in the double bond of fumarylacetoacetic acid with hydrogen, shows almost identical spectrum with that of triacetic acid.

Fumarylpyruvic acid, as mentioned above, shows very similar absorption spectra to that of fumarylacetoacetic acid, forms the conjugated double bond system and was presumed that most of this acid exists in *mono*-enol form even in acidic side.

W. E. Knox et al.[9] reported that maleylacetoacetic acid which absorbs strongly in alkaline solution only, is initial product of homogentisic acid oxidation. In the gentisic acid oxidation, *cis*-isomer of fumarylpyruvic acid is expected to exist prior to fumarylpyruvic acid which is the *trans*-form, since the original configuration around these carbon atoms in the benzene nucleus, gentisic acid, is necessary of *cis* form. The result of spectrophotometrical assay indicates quite enough that meleylpyruvic acid, *cis* isomer of fumarylpyruvic acid, is the direct ring cleavage product. It might be impossible to isolate this acid in a pure state, since this acid converts spontaneously into *trans*-form, particularly, when it is allowed to stand in acidic side. *Cis-trans* isomerization between both isomeric acids is considered to be catalyzed by enzymes included in crude extracts, since the purified enzyme produces maleylpyruvic acid and the dialyzed crude enzyme accumulates fumarylpyruvic acid respectively.

The evidence for existence of two intermediates, the one is fumarylpyruvic acid obtained in a crystalline form and identified, and the other is maleylpyruvic acid recognized spectrophotometrically, establishes the oxidative metabolism of gentisic acid by *Pseudomonas ovalis* S-5 as follows.



6) A. Meister, *J. Biol. Chem.*, **178**, 577 (1949).
7) A. Meistes and J. P. Greenstein, *J. Biol. Chem.*, **175**, 573 (1948).
8) R. G. Ravdin and D. I. Crandall, *J. Biol. Chem.*, **189**, 137 (1951).
9) W. E. Knox and S. W. Edward, *J. Biol. Chem.*, **216**, 489 (1955).

SUMMARY

1) One of the presumed intermediates of the gentisic acid oxidation was obtained in a crystal line form using dialyzed crude gentisic acid

oxidase, free from fumarylpyruvic acid hydrolyase, from the cells of *Pseudomonas ovalis* S-5.

2) From the results obtained, the crystalline intermediate was identified as fumarylpyruvic acid, which was splitted by crude extracts into pyruvic and fumaric acids.

3) Maleylpyruvic acid, the initial product of gentisic acid oxidation, was recognized spectrophotometrically in the reaction mixture using purified enzyme. And the pathway of gentisic acid oxidative metabolism was established.

4) Colorimetric determination of fumarylpyruvic acid is also described.

The authors wish to express their sincere thanks to Honorary Prof. K. Sakaguchi for his continued guidance throughout this research.

Betaine as a Growth Factor for *Pediococcus soyae**

Part VIII. Studies on the Activities of Bacteria in Soy Sauce Brewing

By Kenji SAKAGUCHI

Besides P-factor[1], *Pediococcus soyae*[2,3] requires betaine (glycine-betaine) as a specific growth promotant. The maximal growth is obtained with the supplementation of both betaine and P-factor to the synthetic medium, while betaine only gives the half-maximal growth. When the organism is cultured in 18% salted media, the addition of both betaine and P-factor is essential for the occurrence of growth. Thus, betaine is requisite for *Ped. soyae* as a growth promoting factor and also as a factor bestowing the osmotelerant ability.

Pediococcus soyae is characterized by three eminent specificities, namely, by its acid-sensitivity[2], by its osmotolerant character to grow even in 24~26% salted media[1,2], and by its unique nutritional requirements[1]. This organism does not grow in the ordinary syntheic medium for lactic acid bacteria, and requires two additional growth factors. One of them, which was provisionally named as P-factor in the preceding papers, is found to be included in partial hydrolysates of Hammarsten's milk casein prepared with hydrochloric acid or various crystalline proteinases. The other, S-factor, is found to be present in Difco's yeast extract, beef extract, heart infusion broth, in soy sauce, and in the water extract of *Aspergillus soyae* mycelium. The maximal growth is, however, obtained by the addition of both of the two factors; P-factor alone, giving rise to only the half-maximal growth.

This report deals with the betaine requirement of *Ped. soyae* and the fact that the S-factor and betaine are probably identical. Some results obtained from the examination of specific nutritional requirements of the organism under salt-tolerant conditions are also described.

METHODS

I. Assay procedure.

The basal synthetic medium employed was essentially the same as that described by Snell and others[4] with the following alteration. The supplementation was choline 2 mg/l, inositol 2 mg/l and leucovorin 10 γ/l was made. Pyridoxal was omitted because it is replaceable with pyridoxine. L-Leucine and L-methionine were each substituted by two-fold amounts of DL-leucine and DL-methionine.

Stock cultures of *Ped. soyae* strain d 2 were maintained on 7% salted yeast extract-peptone-glucose-agar stabs and transferred monthly. Inocula were grown in the following medium; yeast extract (Difco) 0.3%, Polypeptone (Takeda) 1.0%, glucose 1.0%, sodium chloride 0.5%, sodium acetate trihydrate 3.3% and dipotassium phosphate 0.5%, pH 7.0. After four days of incubation at 30°C, the cells were centrifuged, washed four times with 0.9% potassium chloride solution, and diluted to give a final optical density of 0.07 at 660 mμ. Five-ml culture fluids containing appropriate supplements were prepared in the usual manner[1], autoclaved for fifteen minutes at 120°C, cooled, incubated with one drop per

*Reprinted by permission of Agr. Chem. Soc. Japan from Bull. Agr. Chem. Soc. Japon, Vol. 24, No. 5.

1) Kenji Sakaguchi, This Bulletin, **23**, 438, 443 (1959).

2) Kenji Sakaguchi, This Bulletin, **22**, 353 (1958); **23**, 22, 100 (1959); *Rep. Noda Inst. Sci. Res.*, **2**, 40 (1958).

3) K. Yamazato and H. Iizuka, *J. Agr. Chem. Soc. Japan*, **33**, 379, 383 (1958); *J. Gen. Appl. Microbiol.*, **5**, 58 (1959).

4) E. E. Snell, *Nutrition Abstr. & Rev.*, **16**, 497 (1946~47); G. Tamura et al., *J. Agr. Chem. Soc. Japan*, **26**, 464, 474 (1952).

tube of the cell suspension described above and incubated at 30°C. Growth was estimated photometrically at 660 mμ in a spectrophotometer. When the cells were cultured in 18% salted broth, and washed with a solution containing 18% sodium chloride, 1.0% glucose, and 3.3% sodium acetate trihydrate, pH 6.0. This solution is favourable for keeping the viability of the inocula grown in salted media.

TABLE I. COMPOSITION OF THE BASAL-SYNTHETIC MEDIUM

DL-Ala	200 mg/l
DL-Asp	400
L-Glu	500
L-Arg·HCl	200
L-Lys·HCl	200
L-His·HCl	100
DL-Ileu	200
DL-Met	200
DL-Phe	200
L-Pro	100
DL-Thr	200
L-Tyr	100
DL-Val	200
DL-Try	100
L-CyS	100
DL-Ser	100
Gly	100
L-Len	100
Glucose	20 g/l
Na-acetate·$3H_2O$	33
K_2HPO_4	500 mg/l
KH_2HO_4	500
$MgSO_4 \cdot 7H_2O$	200
$FeSO_4 \cdot 7H_2O$	10
$MnSO_4 \cdot 4H_2O$	10
NH_4Cl	3 g/l
Adenine sulphate	10 mg/l
Guanine HCl	10
Uracil	10
Xanthine	10
Riboflavine	2
Thiamine·HCl	1
P.A.B.A.	1
Pyridoxine·HCl	1
Ca-*dl*-Pantothenate	1
Nicotinic acid	1
Biotin	10 γ/l
Folic Acid	10
Choline	2 mg/l
Inositiol	2
Leucovorin	10 γ/l

pH 7,0, Pyridoxal is omitted.

RESULTS

I. Requirements of both P- and S-factors in salted environments.

When cultured in unsalted media *Pediococcus soyae* shows half-maximal growth with the supplementation of P-factor alone, and maximal growth is observed by the addition of both P- and S-factors[1]. In 18% salted media, however, nothing was known on nutritional requirements. The results shown in FIG. 1 indicate that, under

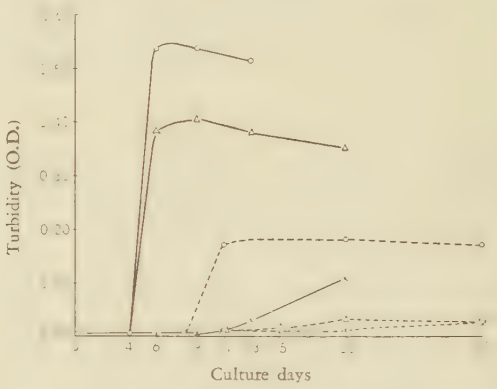


FIG. 1. Nutritional Requirements of *Ped. soyae* in Salted and Unsalted Media.

the salted environment, no growth was observed by the mere addition of P-factor (Polypepton Takeda 1.0%) and the addition of both P- and S-factors was absolutely needed for the occurrence of growth. In the cultures with heavily salted media a longer lag period of more than six days resulted, and the final growth was also poorer.

II. Detection of the effective substance amongst various vitamins and growth stimulants.

Seventy-six vitamins and growth stimu-

lants[5~18] were collected and their effects on *Ped. soyae* were examined. The inspection was made after four days' culture in unsalted media, and after fifteen days' culture in 18% salted media supplemented with P-factor. Amongst

TABLE II. EFFECT OF GROWTH PROMOTANTS ON *Ped. soyae*

	μg/ml	NaCl 0.02%	NaCl 18% polypepton 1.0%
none		0.01	0.00
polypepton	10.000	0.53	0.05
polypepton + yeast-extract		0.76	0.14
polypepton + beef-extract		——	0.15
betaine HCl	30	0.45	0.00
betaine HCl + polypepton		0.67	0.17
ergosterol	10	0.00	0.04
cholesterol	//	0.00	0.02
progesterone	//	0.00	0.02
α-tocopherol	//	0.00	0.08
ATP	//	0.03	0.04
FMN	//	0.02	0.04
FAD	//	0.03	0.05
DPN[14]	//	0.02	0.05
TPN	//	0.03	0.05
coenzyme A	//	——	0.05
yeast RNA	//	0.03	0.05
spleen DNA	//	0.00	0.04
adenosine[15]	//	0.02	0.05
adenylic acid	//	0.01	0.04
deoxyadenosine	//	0.01	0.05
deoxyadenylic acid	//	0.01	0.04
cytidine	//	0.01	0.04
cytidylic acid[16]	//	0.03	0.04
deoxycytidine	//	0.02	0.04
deoxycytidylic acid	//	0.01	0.04
guanosine	//	0.02	0.04
guanylic acid	//	0.03	0.04
deoxyguanosine	//	0.02	0.06
deoxyguanylic acid	//	0.03	0.04
uridine	//	0.04	0.05
uridylic acid[16]	//	0.01	0.04
thymine	//	0.01	0.06
thymidine[17,18]	//	0.03	0.05
thymidylic acid	//	0.04	0.04
Panvitan solution	1000	0.00	0.04
VB_{12}[19]	10	0.00	0.06
VK_3	10	0.00	0.07
nyacin amide	//	0.02	0.05
ascorbic acid	100	0.01	0.07
mevalonic acid (synth.)[20,21]	10	0.02	0.04
// (natural)	//	——	0.06
lipoic acid[22]	//	0.01	0.05
orotic acid[23,24]	//	0.03	0.05
oleic acid[25,26]	//	0.03	0.06
DL-desthiobiotine	//	0.01	0.03
DL-pantoyl lactone	//	0.03	0.05
taurine	//	0.03	0.05
tween 80[25,27]	200	0.04	0.05
N-acetyl glucosamine[28~30]	100	0.01	——
β-alanine	10	0.02	0.05
D-glutamic acid[31]	100	0.03	0.04
diiodotyrosine	10	0.01	——
thyroxine	//	0.02	——
insulin	100	0.01	0.07
glutamine	//	0.01	0.04
asparagine	//	0.01	0.04
glutathione	//	0.01	0.04
Pro-Leu anhydride[32]	//	——	0.03
Pro-Val anhydride[32]	//	——	0.03
Pro-Leu	//	——	0.03
Pro-Val	//	——	0.03

5) E.E. Snell, *Ann. Rev. Microbiol.*, **3**, 97 (1949).
6) W.A. Krehl and S.J. Kiao, *ibid.*, **5**, 129 (1951).
7) J.L. Stokes, *ibid.*, **6**, 29 (1952).
8) V.H. Cheldelin and T.E. King, *ibid.*, **7**, 113 (1953).
9) D. Hendlin, *ibid.*, **8**, 47 (1954).
10) E.L.R. Stockstad et al., *ibid.*, **9**, 111 (1955).
11) L.D. Wright, *ibid.*, **10**, 141 (1956).
12) B. Magasanik, *ibid.*, **11**, 221 (1957).
13) B.M. Guirard, *ibid.*, **12**, 247 (1958).
14) K. Kitahara and A. Ôbayashi, *J. Agr. Chem. Soc. Japan*, **33**, 497 (1959).
15) T. Fukami, Lecture given on July 4th, 1959 at the First Meeting of the Symposium on Microbioassay.
16) M. Ikawa and J.S. O'Bar, *J. Bact.*, **71**, 401 (1956).
17) E.A. Felton and C.F. Niven, *J. Bact.*, **65**, 482 (1953).
18) E. Kitay and E.E. Snell, *ibid.*, **59**, 727 (1950); **60**, 49 (1950).
19) I. Kusaka and K. Kitahara, *J. Agr. Chem Soc. Japan*, **33**, 703 (1959).
20) G. Tamura, This Bulletin, **21**, 202, 394 (1957); **22**, 202 (1958); *J. Agr. Chem. Soc. Japan*, **32**, 701, 707, 778, 783 (1958).
21) D.E. Wolf et al., *J. Amer. Chem. Soc.*, **78**, 4499, 5273 (1956); **79**, 1486 (1957).
22) L.J. Reed et al., *Science*, **114**, 93 (1951).
23) I. Crawford et al., *J. Biol. Chem.*, **226**, 1093 (1957).
24) L.D. Wright et al., *Proc. Soc. Exptl. Biol. Med.*, **84**, 716 (1953).
25) Y. Tani and S. Tatsumi, *J. Ferment. Technol.*, **36**, 311, 317 (1958).
26) K. Hofmann, et al., *J. Biol. Chem.*, **228**, 349 (1957).
27) L. Gerschenfeld, *J. Am. Pharm. Ass. Ed.*, **42**, 187 (1953).
28) P. Georgy, R. Kuhn et al., *Am. J. Disease Children*, **84**, 484 (1952); **85**, 632 (1953).
29) R. Kuhn, *Angew. Chem.*, **64**, 493 (1952).
30) S. Pope et al., *Arch. Biochem. Biophys.*, **68**, 362 (1957).
31) S.A. Koser and J.L. Thomas, *J. Bact.*, **73**, 477 (1957).
32) Y. Koaze, This Bulletin, **21**, 197 (1957); **22**, 91, 98, 238 (1958).

γ-amino butyric acid	//	0.01	0.04
oxaloacetic acid	100	0.01	——
Ba-glucose-6-phosphate	//	——	0.04
Ba-hexose-1,6-diphosphate	//	0.02	0.05
Na-β-glycerophosphate	//	0.01	0.05
dihydroxyacetone	//	0.01	——
fumaric acid	//	0.00	——
lactic acid	//	0.00	——
succinic acid	//	0.00	——
glycolic acid	//	0.00	——
malic acid	//	0.00	——
citric acid	//	0.01	——
DL-glyceraldehyde	//	0.01	——
Ba-phosphoglycerate	//	0.01	——
pimelic acid	10	0.01	0.04
L,L-diamino pimelic acid[33,34]	//	0.01	0.05
L,D-diamino pimelic acid	//	0.01	0.05
D,D-diamino pimelic acid	//	0.00	0.06
rutin	//	0.03	0.05

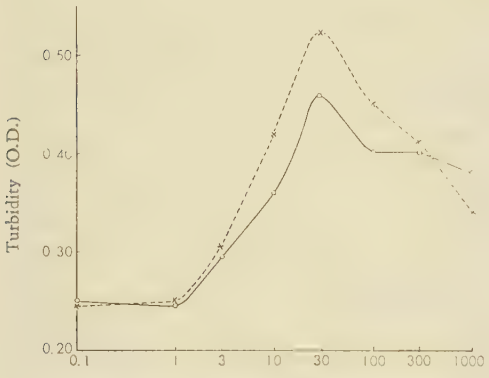


FIG. 2. Optimal Concentration of Betaine Hydrochloride.
—○— 9 days incubated.
- - -×- - - 10 days incubated.

various growth promotants only betaine was found to be effective in both salted and unsalted media.

III. Optimum concentration of betaine hydrochloride.

To the unsalted synthetic medium, betaine hydrochloride was added in concentrations of 0.1, 1.0, 3.0, 10, 30, 100, 300, and 1000 mg/l and the growth response was measured after nine and ten days of culture. Fig. 2 shows that the best growth was obtained with the addition of 30 mg betaine hydorochloride per litre. However, even in this optimum concentration, the supplement of betaine alone resulted in the half-maximal growth. After nine days of culture, the synthetic media attained the final growth of 0.25 optical density, the supplement of 1.0% Polypepton 0.43, betaine hydrochloride 30 mg/l 0.46, while media added with Polypepton 1.0% and yeast extract 0.3% attained the optical density of 0.64. It is thus approvable that besides betaine, another growth factor is necessary for the maximal growth of *Ped. soyae.*

IV Effects of various amines and related substances with betaine.

Betaine is known as an intermediate metabolite of choline, which degrades through the path of betaine, dimethyl-glycine, sarcosine, reaching to glycine. The liberated methyl group are reported to make transmethylation reaction with various substances like homocysteine[35]. However, the substances relating the path were all ineffective to *Ped. soyae* except betaine (Table III).

On the nutritional effects of amines, Herbst and Snell[36,37] first reported that spermine, spermidine, putrescine or agmatine were essential growth factors for *Haemophilus parainfluenzae.* Momiyama and Miyaki[38] reported that putrescine, agmatine or arcaine were required by *Escherichia*, *Aerobactor* and *Salmonella.* That ethanolamine was effective for stimulating the growth of *Proteus morganii* was also reported by the same authors[39]. Concerning the lactic acid bacteria, it was observed by Kihara and Snell[40] that the growth of *Lactobacillus casei* was markedly accelerated by the co-operation of strepogenin and spermine.

As for the nutrition of *Ped. soyae*, choline is

33) L.E. Rhuland., *J. Bact.*, **73**, 778 (1957).
34) P. Meadow et al., *Biochem. J.*, **66**, 270 (1957).
35) D.M. Greenberg, Chemical Pathways of Metabolism, vol. **2**, 117 (1954).
36) E.J. Herbst and E.E. Snell, *J. Biol. Chem.*, **176**, 989 (1948); **181**, 47 (1949).
37) E.J. Herbst, E.B. Glinos and L.H. Amunndsen, *Federation Proc.*, **11**, 229 (1952); *J. Biol. Chem.*, **141**, 47 (1955).
38) H. Momiyama, *Nippon Eiseigaku Zasshi*, **10**, 42 (1956); **11**, 291 (1957).
39) H. Momiyama, *ibid.*, **11**, 296 (1957).
40) H. Kihara and E.E. Snell, *Proc. Natl. Acad. Sci.* **43**, 867 (1957).

quite ineffective because it is included as a constituent in the basal synthetic medium. Other 22 amines listed in Table IV were also not effective in inducing the growth of *Ped. soyae* when tested at the concentration of 10 mg/l.

Table III. Effect of Betaine and its Related Substances

	O.D.	N/20 NaOH ml
None	0.13	2.14
Betaine HCl 20mg/l	0.31	4.20
Lecithine 100mg/l	0.17	2.15
Acetyl choline chloride 20mg/l	0.14	2.30
Sarcosine 20mg/l	0.13	2.05
Procaine HCl 20mg/l	0.13	2.00
Putrescine 20mg/l	0.12	2.26
Dimethyl-glycine HCl 100mg/l	0.12	2.23
// 10mg/l	0.13	2.15
// 1mg/l	0.13	2.00
Dimethyl-glycine-methyl ester 100mg/l	0.13	2.18
// 10mg/l	0.12	2.05
// 1mg/l	0.13	2.22

After 17 days of incubation, the cell growth was assayed turbidimetrically and acidimetrically.

Table IV. Effect of Various Amines

	4 days	9 days
None	0.01	0.14
Betaine	0.45	0.47
Trimethylamine	0.01	0.15
Monoethylamine	0.01	0.14
Diethyl amine	0.01	0.15
Triethyl amine	0.01	0.15
Monoethanol amine	0.01	0.16
Diethylamino ethanol	0.02	0.14
Tetramethylammonium hydroxyde	0.02	0.06
β-phenyl-ethylamine	0.01	0.14
Dicyclohexyl amine	0.01	0.14
Tyramine HCl	0.01	0.15
Putrescine 2HCl	0.01	0.14
Cadaverine 2HCl	0.01	0.15
Histamine 2HCl	0.00	0.15
DL-Ornithine HCl	0.00	0.14
Agmatine H_2SO_4	0.01	0.15
Tryptamine	0.01	0.15
Isoamylamine	0.00	0.15
Spermine	0.01	0.14
Spermidine	0.00	0.14
Ergothioneine	0.00	0.13
Creatine[41,42]	0.00	0.14
Creatinine	0.00	0.14
Mixture of all	0.00	0.00

The table shows the cell growth by optical density.

V S-factor's effects represented by betaine.

The fact that the supplementation of basal medium with betaine only resulted in the half-maximal growth offers an another problem whether the growth stimulating effect of P- or S-factor could be wholly attributed to that of betaine. In order to examine this, experiments were conducted as follows. *Ped. soyae* was grown in both 18% salted and unsalted media supplemented with P-factor (Polypepton) only, with P-factor and betaine, with P-factor and S-factor (yeast extract), and with betaine only. Results given in Fig. 3 shows that maximal growth response was obtained by the addition of both P-factor and betaine as well as in the case of P-factor plus S-factor under the both environments of salted and unsalted media. Thus the effect of betaine is identical with that of S-factor. It was also noted that the single supplement with either betaine or P-factor to 18% salted media showed almost null growth even after thirty days of incubation.

VI. Existence of betaine in yeast extract and beef extract.

As S-factor rich substances, yeast extract, beef extract, heart infusion broth (Difco)[3], unpasteurized soy sauce, and water extract of *Aspergillus sojae* mycelium[1] have been described in former reports. Sumi[43] reported the existence of a considerable amount (0.9 g/100 g) of betaine in spores of *Aspergillus*. Udō[44] in his laborious work on the constituents of soy sauce also found betaine with its sweet taste. Although betaine is widely distributed in plants[45], no information has been reported on its presence in yeast extract and beef extract. It is also questionable that the growth promoting substance in yeast extract or beef extract is only betaine.

The experiments were conducted chromato-

41) R.H. Nimmo-Smith and G. Appleyard, *J. Gen. Microbiol.*, **14**, 336 (1956).
42) G. Appleyard and D.D. Woods, *ibid.*, **14**, 351 (1957).
43) M. Sumi, *J. Agr. Chem. Soc. Japan*, **2**, 866 (1925)
44) S. Udo, *ibid.*, **7**, 852 (1931); **8**, 675 (1932).
45) M Guggenheim, Die Biogenenaminen, p. 193 (1940).

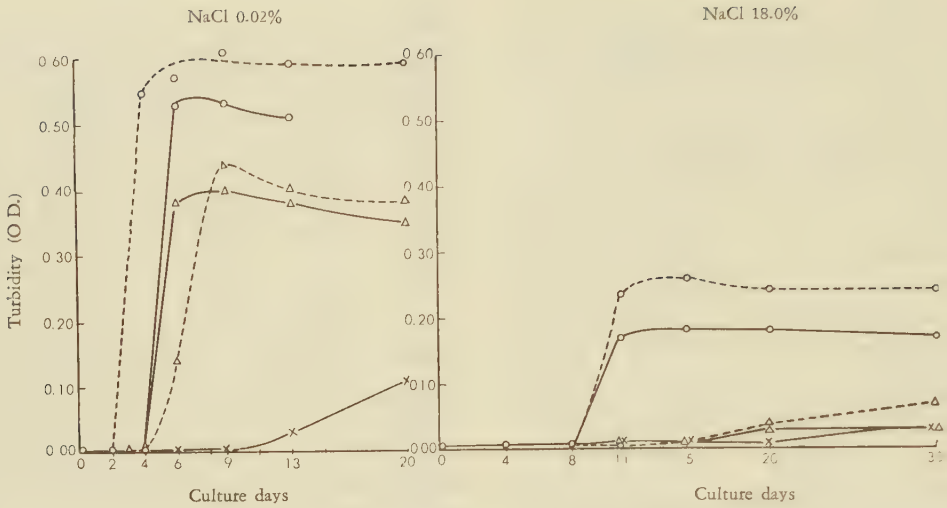


FIG. 3. S-factor Effect Represented by Betaine.

—×— Synthetic medium
—△— +Polypepton 1.0%
—○— +Polypepton 1.0% & yeast extract 0.3%
- - -△- - - +betaine 30 mg/l
- - -○- - - +betaine 30 mg/l Polypepton 1.0%

graphically to identify the biologically active spot and the spot of betaine on paper. Yeast extract and beef extract (Difco) were extracted with ethanol, the soluble fraction and the residue were each spotted on the lower tips of paper strips (Tōyō Roshi No. 53) and developped for about thirty hours at 20°C using the solvent of *n*-butanol, acetic acid and water mixed in the ratio of 4 : 1 : 2. To detect the microbiologically active spot, the developped and dried paper strip was cut in each 1 cm, put into cotton plugged test tubes, poured by the 5 ml of basal synthetic medium, and their growth promoting acitivities were assayed as usual. The colour spot of tertiary amines and tetrary ammonium salts including choline and betaine was detected by spraying Dragendorff's reagent. The samples added with betaine were also chromatographed to calibrate the R_F of betaine in crude mixtures. Fig. 4 shows that the growth stimulants are rather ethanol soluble, Dragendorff positive spots and the spot of mixed betaine are included in the area of biologically active spots. The active spots which do not coincide with the spots of tertiary amines may be partly due to the action of contaminating P-factor. Such phenomena is rather distinct in the case of yeast extract. In general, at least a part of the S-factor substances in yeast extract or beef extract are attributable to betaine contained in them.

DISCUSSION

Pediococcus soyae is a very specific lactic acid bacterium in the respect of its high osmotolerant character growing even in 24~26% salted media[1]. This organism grows only very poorly in the ordinary synthetic medium employed for bioassay[5], and requires two different specific growth factors for maximal growth. One of them, provisionally named as S-factor in former reports, is a constituent of Difco's yeast extract, beef extract, heart infusion broth, unpasteurized Japanese soy sauce, etc.. In this report it is elucidated that the action of S-factor was perfectly replaced by the addition of betaine hydrochloride 30 mg/l. It was therefore observed that at least one of the active constituents in the yeast extract or beef extract was betaine. However, the

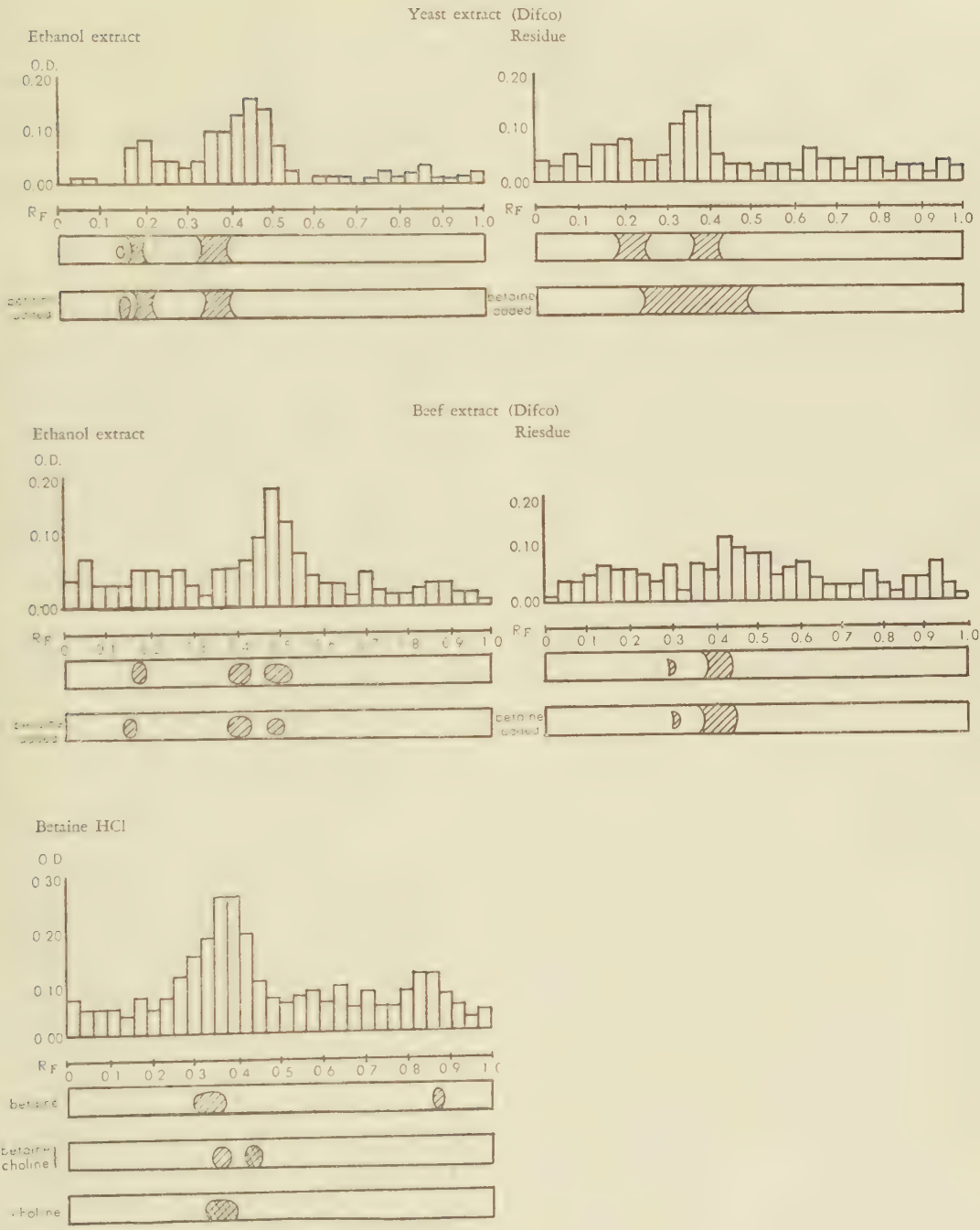


FIG. 4. Bioautograms of Yeast Extract, Beef Extract and Betaine-HCl, and their Paper Chromatograms Sprayed by Dragendorff's Reagent.

problem that some other growth stimulating substances might exist in them is still remained to be clarified. The optimum concentration of betaine hydrochloride as high as 30 mg/l also sustains this question because it is too high as compared with the required amounts of ordinary vitamins and it almost nears to the level of amino acids or other substrates.

Betaine is a usual component in plants[45], and it is expected that the substance may play some roles in their metabolism and physiology. It has been known that betaine is an intermetabolite of choline, and it reacts as a substrate of transmethylation. It has an effect as a lipotropic factor for the prevention of animal fat liver, and also as a growth factor substitutable with choline. However, betaine is useless, in contrast to choline, for the prevention of chick's perosis and for its growth stimulation[46,47]. As for the effects of betaine against *Ped. soyae*, the growth promoting effect and the osmotolerancy bestowing effect, are the unique faculty of betaine because no other metabolically related substances such as choline, dimethyl glycine, sarcosine or methionine are effective. It is supposed from these facts that betaine is effective for *Ped. soyae* not through the metabolic path ever known, but may be directly effective for maintaining the semi-permeability of the cell membrane in co-operation with P-factor.

The author wishes to express his hearty thanks to Prof. T. Asai and Prof. K. Kitahara of the Institute of Applied Microbiology, University of Tokyo for their kind guidances. He is greatly indebted to Prof. K. Miyaki and Assist. Prof. M. Hayashi of Chiba University for their kind advices and gift of dimethylglycine. Generous supply of many precious vitamins and growth factors were made by Assist. Prof. G. Tamura and Mr. T. Fukami of University of Tokyo. The author's thanks are also due to the kind advices given by Dr. Mogi and members of this institute. He is especially grateful to Mr. T. Kuramochi for his earnest and able assistance.

46) A.D. Welch, *J. Nutr.*, **40**, 113 (1950).
47) T.H. Jukes and A.D. Welch, *J. Biol. Chem.*, **146**, 19 (1942).

Studies on L-Valine Fermentation*

Part I. Production of L-Valine by *Aerobacter* Bacteria**

By Zenzirō Sugisaki

A marvelous outbreak in the field of the so-called "Amino acid Fermentation" has first been performed by Japanese workers such as Mr. Kinoshita et al.[1] in observations concerning L-glutamic acid fermentation. The basic concept is now successfully extended to microbial methods for the preparation of essential amino acids, for instance, L-lysin[2] and L-tryptophane[3], etc.

Meanwhile, L-valine is reported to be detected in a minute amount inside the cell or in the surrounding medium by many investigators[7~9] in detailed studies on biosynthesis, and more recently, Asai et al.[4] and Kinoshita et al.[1] have further pointed out the presence of a trace-amount of valine detectable with other amino acids in the culture medium in case of amino acid fermentation caused by some microbes. However, the production of valine in a high level has never been described, until Udaka et el.[5] recently reported on the production of L-valine by some microorganisms, especially, *Paracolobactrum coliforme*, which accumulates 7~8 mg of valine per ml of broth after 48 hours' incubation by shake culture.

From an independent standpoint of these workers, the present author has undertaken extensive studies on the subject and it has been found that L-valine can be produced by some newly isolated specific strains from carbohydrates and ammonia sources in the culture broth at a high level of 12~13 mg per ml of broth. The author thus investigated the possibility of formation of L-valine fermentation.

In practice, it is worthy to take advantage of a method to readily produce an optically active L-valine product, due to the fact that it is still prepared by means of resolution of chemical synthetics, DL-isomers, or the separation from protein hydrolysates.

EXPERIMENTAL

Isolation and Screening of L-Valine Producing Bacteria

Isolation of organisms ; Performed in the usual manner.

Screening experiments ; The composition of the medium used in the screening test was as follows : 10% glucose, 1.2% NH_4Cl, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$. Ten ml of the medium placed in a test tube (D=23 m/m) was inoculated with one loopful of the organisms from each slant culture, adding 3~4% $CaCO_3$ sterilized separately, and incubated with shaking culture employing a reciprocal shaker at 350 r.p.m., amplitude of vibration, 2 cm. The amino acids produced in the broth were analysed after three days' culture of bacteria.

Analytical methods ; A routine identification and rough

*Reprinted by permission of the Inst. Appl. Microbiol., Univ. of Tokyo, from J. Gen. Appl. Microbiol. Vol. 5, No. 3.

**This work was presented at the meeting of the Agricultural Chemical Society of Japan held at Tokyo University on Feb., 28, 1959.

1) S. Kinoshita, S. Udaka and M. Shimono, *J. Gen. Appl. Microbiol.*, **3**, 193 (1957).

2) S. Kinoshita, K. Nakayama and S. Kitada, *J. Gen. Appl. Microbiol.*, **4**, 128 (1958).

3) K. Aida, T. Asai, T. Kajiwara and K. Iizuka, *J. Gen. Appl. Microbiol.*, **4**, 200 (1958).

4) T. Asai, K. Aida, K. Oishi, *J. Ferm. Assoc. (Japan)* **15**, 1 (1957).

5) S. Udaka, K. Tomizawa and S. Kinoshita, Lecture delivered on the meeting of Agricultural and Chemical Society in Japan, Oct. 25, 1958.

6) S. M. Partridge and R. C. Brimely, *Biochem. J.* (London) **48**, 313 (1951).
S. M. Partridge, *Biochem. J.* (London) **44**, 521 (1949).

7) M. Strassman, A. J. Thomas and S. Weinhouse, *J. Am. Chem. Soc.* **77**, 1261 (1955).

8) H. E. Umbarger and B. Brown, *J. Biol. Chem.*, **233**, 1156 (1958).

9) R. P. Wagner, A. Bergquist and H. S. Forrest, *J. Biol. Chem.*, **234**, 99 (1959).

quantitative estimation of the amino acids were made by the paper chromatographic procedure carried out in the usual manner. For the screening test, 2 μl of the broth was spotted on the filter paper, Toyo No. 50, which was developed with use of *n*-butanol-acetic acid-water (4 : 1 : 1) for about eighteen hours at 20°C. The chromatogram was treated with 0.2% ninhydrin solution in 96% ethanol. Rf-value of valine is about 0.50. Quantitative analysis of L-valine was made by application of the ordinary bioassay technique, using *Leuconostoc mesenteroides* P-60 or *Lactobacillus arabinosus* 17-5. Glucose was determined according to the method of Fehling-Lehmann-Schoorl, and ammoniacal nitrogen by CONWAY'S micro diffusion analysis[13]. Turbidity and pH were measured by a Hitachi Photoelectric Spectrophotometer and Beckman type glass electrode pH-meter respectively.

Results: Numerous known and isolated bacteria were tested in the screening experiments. The valine spot on paper chromatogram was detected in the usual manner which is described above. As already cited by some other investigators, it was frequently found that a considerable number of bacteria produce alanine, glutamic acid, or aspartic acid, and also that a trace amount of valine producing microorganisms are widely distributed not only in some genera of *Enterobacteriaceae* but in other kinds of bacteria and furthermore, in some genera of yeasts, not to mention *Escherichia coli*, *Neurospora crassa*, *Saccharomyces cerevisiae* and *Torulopsis utilis* in agreement with earlier observations[7~9]. However, in most of these cases valine was rathermore a by-product accompanied with other main amino acid products. The amount of valine produced by comparatively specific bacteria ranged from 1~3 mg of ml broth in many strains, and in a few strains far more than 5 mg. However, some specific strains which solely accumulate L-valine in a high level could rarely be found, and most of them belong to the *Coli-Aerogenes* group. Thus the highest level of L-valine accumulation, 8~12 mg per ml broth, was shown by some of the newly isolated strains, falling into the head of Genus *Aerobacter*; NISR-B-151, NISR-B-801 and that group.

Identification of L-Valine Producing Bacteria

Methods: Diagnostic tests were carried out according to "Manual of microbiological methods" by the Society of American Bacteriologists (1957), and referred to "Hand-book of Practical Bacteriology" by Mackie & MacCartney 9th Ed., E. & S. Living Stone, Ltd., Edinburgh and London (1956), "An Introduction to Laboratory Technique in Bacteriology" by Max Levine 3rd Ed., McMillan Co., New York (1954), and "Saikin-Gaku Jisshu Teiyo" by the Institute for Infectious Diseases, Univ. of Tokyo.

Results: In the present table (Table I), a detailed description of two strains of the specific bacteria is presented.

On the basis of these data, comparative studies were conducted in accordance with the descriptions of BERGEY'S manual, 6th and 7th Ed.

1. *Decision of Family:* Both strains are Gram-negative, aerobic or facultatively anaerobic, nonsporulating, small rods, fermenting glucose with production of acid and gas, and produce nitrites from nitrates characteristically. The strains therefore fall under the head of Family *Enterobacteriaceae*.

2. *Decision of Tribe:* Strains ferment lactose, producing acid and gas, but do not produce prodigiosin and protopectinase; therefore they should be classified as Tribe *Escherichieae*.

3. *Decision of Genus:* From the so-called IMViC test (−−++) and gas ratio in glucose decomposition, they should fall under the category of Genus *Aerobacter*.

4. *Decision of Species:* Though strain NISR-B-151 is considered to be *A. cloacae*, since this strain ferments glycerol with the production of acid and no visible gas and liquefies gelatin; but it is differs in some points ragerding characters in type culture –production of hydrogen sulfide, decomposition of sodium alginate, and fermentation of some sugars—so that it is recognized to be *A. cloacae var. sp.* Another strain NISR-B-801, ferments glycerol, producing acid and gas, but does not liquefy gelatin, other properties also being similar to that of the type culture. Thus this strain is identified as one of the typical *A. aerogenes*.

In the above strains, it is an exceedingly specific character to exhibit the strong activity of L-*valine accumulation*, which is more markable than common *Coli-Aerogenes* bacteria, in culture media from carbohydrates and nitrogen sources by shake culture.

L-Valine Production from several kinds of Carbohydrates and Nitrogen sources by Aerobacter.

By shake culture, both strains produce valine not only from glucose and ammonium chloride but also, more or less, from several kinds of carbohydrates and ammonium sources, as shown qualitatively in Table II and III. In all of the author's experiments, methods similar to those described before were applied except in case of media noted in each table.

1. L-*Valine production from several kinds of Carbohydrates*

Each media contained 2% carbohydrates and 0.7%

TABLE I. CHARACTERS OF L-VALINE PRODUCING BACTERIA

Description	Strain NISR-B-151	Strain NISR-B-801
I. Morphological:		
Vegetative cells	Rods, 0.5~1.0×1.0~2.0 μ, single.	Rods 0.5~0.8×1.0~2.0 μ, single.
	gram negative.	gram negative.
	motile with peritrichous flagella.	non-motile.
	not encapsulated.	capsulated.
Sporangia & End Spores	none.	none.
II. Cultural:		
1. Agar colonies	circular, frequently Rhizoid, thin, opaque with white-center (umbonate), entire.	thick, white, raised, moist, smooth, entire, mucoid.
2. Agar stroke	growth moderate, filiform, glistening, porcelain white.	abundant growth, thick, white, moist, glistening, spreading growth.
3. Gelatin colonies (20°)	thin, smooth, circular, bluish, translucent.	thick, white, moist, smooth, entire.
4. Gelatin stab	slow liquefaction; filiform, napiform to infundibuliform.	no liquefaction; thick, spreading, white, opaque surface growth.
5. Nutrient broth	turbid, thin pellicle, fecal odor not produced.	Turbid, pellicle, abundant sediment.
6. Opt. temperature	30°~38°C.	30°C.
	growth 6~42°C, thermal death point, 65°×10 min. Gas not produced in Eijkman test carried out at 45°~46°C (both).	
7. Opt. pH	pH 6.0~6.8 (limit 4.6~9.0).	pH 6.0~6.8 (limit 4.4~9.0)
8. Relation to free-O_2	Aerobic, facultatively anaerobic.	Aerobic, facultatively anaerobic.
9. Chromogenesis (Potato)	brownish, moist, glistening growth.	thick, yellowish brown, spreading & glistening growth.
III. Physiological:		
1. Litmus milk	acid; coagulation; gas; rapid peptonization.	acid with coagulation; no peptonization.
2. Action on Nitrate (NO_3, NO_2)	+	+
3. Indole production	−	−
4. M.R. Test	−	−
5. V.P. Reaction	+	+ (weak)
6. Utilization of Citrate	+	+
7. H_2S production	−	−
8. Gas Ratio fermented with Glucose	CO_2 : H_2 ≥ 2 : 1	CO_2 : H_2 ≥ 2 : 1
9. Utilization of NH_4'-salt	+	+
10. Utilization of Uric acid	+	+
11. Hydrolysis of Na-hippurate	−	+
12. Decomposition of Na-Alginate	+ (Acid)	−
13. Reduction of Indicator	litmus (+), NR (+)	litmus (+), NR (+)
14. NH_3 production	+	+
15. Catalase	+	+
16. Urease	±	+
17. Fermentation of Sugars (cf. Table 2)		
Glycerol	A	AG
Glucose	AG	AG
Lactose	AG	AG

A: Acid, G: Gas, Media; similar to that in Table II.

NH_4Cl in stead of 10% glucose and 1.2% NH_4Cl, respectively.

It was observed that L-valine is formed from both hexose and most of the assimilative carbohydrates as shown in Table II—from pentoses, oligosaccharides, polyols and polysaccharides.

2. *Utilization of several kinds of Nirogen Sources*

Concentration of each nitrogen source was 0.3% as nitrogen, corresponding to ca 1.2 NH_4Cl.

From Table III it is evident that both strains may be able to utilize several kinds of inorganic and organic nitrogen sources, except nitrites, accompanied with the formation of valine. Further, from some other quantitative experimental results it was found that ammonium salts of strong acids, such as NH_4Cl, $(NH_4)_2SO_4$, NH_4NO_3, etc., are suitable nitrogen sources for this fermentation.

TABLE II. FERMENTATION OF SUGARS & L-VAL. PRODUCTION

	Sugars	Strains: NISR-B-151 Fermentation products A	G	L-Val.	NISR-B-801 Fermentation products A	G	L-Val.
1	Arabinose	+	+	+	+	+	±
2	Rhamnose	+	+	±	+	+	−
3	Xylose	+	+	++	+	+	++
4	Glucose	+	+	++	+	+	++
5	Fructose	+	+	++	+	+	++
6	Galactose	+	+	++	+	+	±
7	Mannose	+	+	++	+	+	++
8	Lactose	+	+	−	+	+	+
9	Sucrose	+	+	−	+	+	+
10	Maltose	+	+	+	+	+	+
11	Trehalose	+	+	++	+	+	±
12	Cellobiose	+	+	++	+	+	±
13	Raffinose	−	−	−	+	+	+
14	Starch	−	−	−	+	+	−
15	Inuline	−	−	−	−	−	−
16	Dextrine	−	−	−	±	−	−
17	Glycerol	±	−	−	+	+	−
18	Arabitol	−	−	−	+	+	++
19	Mannitol	+	+	−	+	+	+
20	Sorbitol	+	+	++	+	+	++
21	Dulcitol	−	−	−	−	−	−
22	Inositol	−	−	−	+	+	+
23	Salicin	±	+	±	+	+	-
24	Aesculin	+	+	−	+	+	+
25	Arbutin	+	+	−	+	+	−
26	α-Methyl-glucose	+	+	−	+	+	±

A: Acid, G: Gas.
1% Sugar solution containing pepton-water and the Ayer-Rupp-Johnson' artificial medium. L-Val. production Media: Sugar 2%, NH_4Cl 0.7%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCO_3$ 1%.

TABLE III. UTILIZATION OF NH_4-SALTS & L-VAL. PRODUCTION

	N-sources	NISR-B-151 Growth	L-Val.	N1SR-B-801 Growth	L-Val.
1	NH_4Cl	++	++	++	++
2	$(NH_4)_2SO_4$	++	++	++	++
3	$NH_4 \cdot H_2PO_4$	++	±	++	+
4	$(NH_4)_2\ HPO_4$	++	+	+	+
5	$NH_4 \cdot NO_3$	++	++	++	++
6	$K \cdot NO_3$	++	+	++	±
7	$Na \cdot NO_3$	++	++	++	±
8	$K \cdot NO_2$	−	−	−	−
9	$Na \cdot NO_2$	−	−	−	−
10	NH_4–Acetate	+	+	++	++
11	$(NH_4)_2$–Tartarate	−	−	++	±
12	NH_4–Lactate	+	++	++	++
13	Urea	±	−	++	+

Med.: Glucose 10%, NH_4-salts 0.3%N, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%; $CaCO_3$ 3%

3. *Progressive-Fermentation of Aerobacter*

One of the specific strains, *A. cloacae var. sp.* NISR-B-151, showed a high level of L-valine accumulation and an example of the progressive production of L-valine by the strain is shown in Fig. 1.

Medium: 10% glucose, 1.2% NH_4Cl, 0.15% KH_2PO_4, 0.03% $MgSO_4 \cdot 7H_2O$, 10 p.p.m. $MnSO_4 \cdot nH_2O$; 3% $CaCO_3$ (sterilized separately).

Incubation: 50 ml of the medium dispensed in a 500 ml shake flask was inoculated with 2 loopfuls from the slant culture of *A. cloacae var. sp.* NISR-B-151, and reciprocal shake culture (136 r.p.m.) was carried out at 30±2°C. Assay methods are similar to that described in the screening experiment.

After 72 hours' incubation, 12 mg of L-valine was produced from 92 mg of consumed glucose per ml of broth, corresponding to 20% of theoretical yield, as mole by mole ratio from consumed glucose may be formed. Similar results were also observed in cases of *A. aerogenes* NISR-B-801.

Isolation and Identification of L-Valine Produced in the Culture Broth

Isolation of Valine: In order to isolate valine produced in the culture broth, it is a more practical procedure to advantageously employ Ion Exchanger Method (Displacement chromatography using a strongly acidic cation exchange resin) reported by Partridge and Brimely[6]

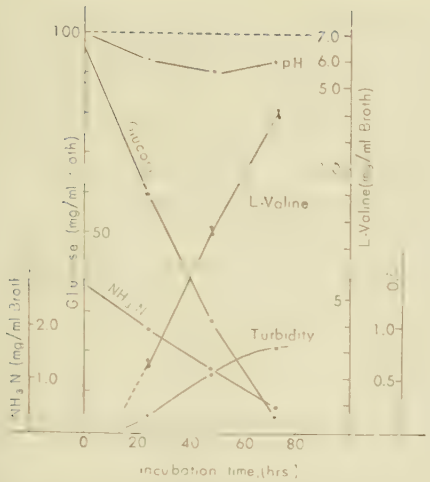


FIG. 1. Chemical changes during Fermentation.
Strain: A. cloacae var. sp. NISR-B-151.
Medium: 9.6% glucose, 1.2% NH_4Cl, 0.15% KH_2PO_4, 0.03% $MgSO_4 \cdot 7H_2O$, 10 p.p.m $MnSO_4 \cdot nH_2O$ (pH 6.6)
Conditions, noted in the text.

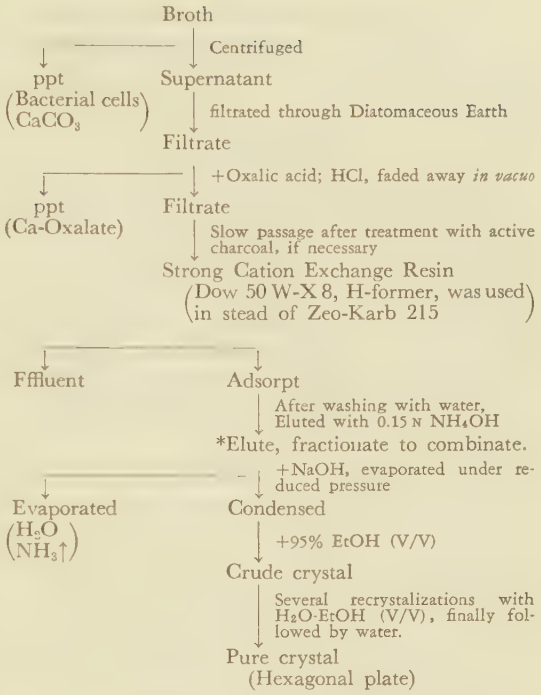


FIG. 2. Isolation of Valine from culture broth.
* Each of these various fractions was tested individually by paper chromatography, and fractions containing valine were combined and evaporated to condensed one under reduced pressure under a weakly basic with driving out ammonia at the same time.

than copper-salt separation or butanol-extraction.

In Fig. 2, the outlines of substantial treatment of the broth and isolation of valine are presented. The isolated crystals, magnificient hexagonal flat-plate, were dried up under reduced pressure in a desiccator and further desiccated by Abderhalden's apparatus.

Identification of L-*Valine*

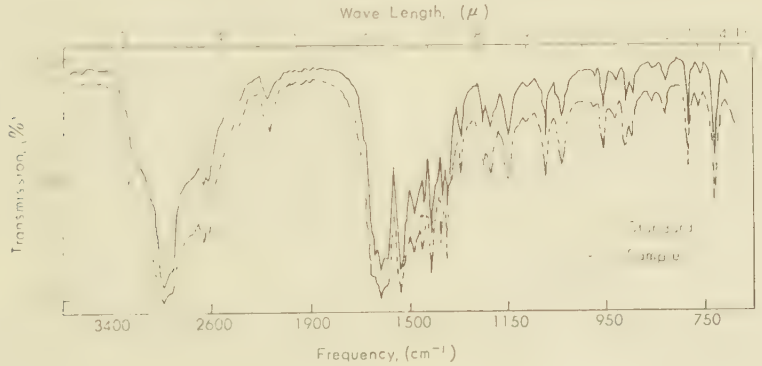
Subsequent to these operations, the crystals were then subjected to the following examination, i.e., identification with L-valine by physical, chemical and biological procedures.

(i) Paper-Partition Chromatography;
Rf, 0.49 (n-butanol-acetic acid-water =4 : 1 : 5, upper layer)
0.81 (phenol-water=4 : 1)

(ii) M.P., 315° (Decomposition; Lit., 315°)
147° as p-Toluensulfonyl derivatives. (auth., 147°; no depression)

(iii) Elementary Analysis;

	C	H	N
Found:	51.18	9.42	11.80
Calc'd. for $C_5H_{11}O_2N$:	51.26	9.45	11.96



(iv) $[\alpha]_D^{20}=+28.4°$ (6N HCl, C 3.4; Lit 28.8°)

(v) Bioassay by *Lact. arabinosus* 17-5 and *Leuc. mesenteroides* P-60 showed to be L-valine.

(vi) Infrared Spectra;

DISCUSSION

It has been observed that the accumulation of L-valine by the specific strains occurs when the organisms are in growth, and that a sole amino acid or only a few kinds of amino acids are produced. Therefore the production of valine may be easily accounted in the absence of autolysis, in which case various kinds of amino acids are expected to be detectable.

Recently, in detailed studies of valine metabolism performed by many workers[7~9], it was recognized that α-acetolactate is the first precursor in L-valine biosynthesis. Thence, together with the early informations[10~14], it was clarified that the pathway of valine biosynthesis appears to be shown in the presented shema diagramed with a solid line in Fig. 3, though some difference in detail was observed between yeast[7] and *E. coli*[8] or *Neurospora crassa*[9].

From the point of view concerning intermediates and chemical changes occurring in this fermentation, similar suggestions have been presented; (1) the so-called C_5-precursors are perceptible in broth, and (2) a considerable amounts of pyruvate and acetaldehyde are detectable in culture media in an early stage of fermentation, and further, (3) L-valine is formed from both hexoses and pentoses, especially readily from fructose and xylose. Thence it may be presumed that the dotted line process and the known successive pathway shown by the solid line should be existential in this fermentation.

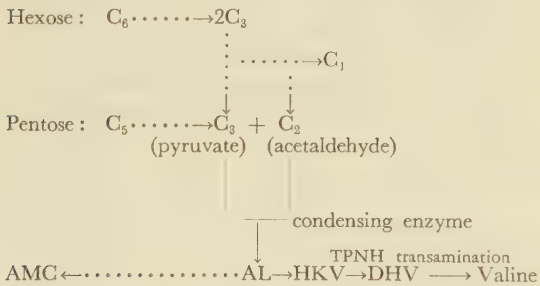


FIG. 3. Known postulated Mechanism of Valine Biosynthesis (→).

Note of abbreviation:
AL; α-acetolactate. HKV; α-keto-β-hydroxy isovaleric acid, the so-called Hydroxy-ketovalin. DHV; α, β-dihydroxy isovaleric acid, the so-called Di-hydroxy valine. KV; α-keto isovaleric acid, the so-called keto-valine. AMC; Acetyl-methyl-carbinol.

Meanwhile, in the course of screening experiments, the author has always held the opinion that L-valine production specificity is noticeable in those microbes which are aerobic or facultatively anaerobic microorganisms, decomposing glucose with the production of acid and gas, and are characteristically positive in the modificated Voges-Proskauer reaction, provided Barritt's modification is applied to the culture media after addition of hot sulphuric acid and polypeptone. In fact, almost all microbes possesing these three properties are able to form valine to a certain extent under similar conditions previously described. Then, it can be properly comprehended, in a broad sense, on the basis of the correspondence between the theory and observations, that the positive reaction in the modificated V-P test is, in general, due to the presence of α-acetolactate rather than acetyl-methyl-carbinol *in this fermentation* broth. Hence L-valine producibility is not always a common property solely observed in *Enterobacteriaceae*. The specific strains which produce a large amount of valine should be endowed with a strongly active enzyme system in this pathway, especially, cleavage enzymes of carbohydrates, α-acetolactate forming enzyme, reductive enzyme requiring TPNH, and transaminase. A detailed study on the mechanism of this fermentation is now in progress.

SUMMARY

1. A screening test for L-valine producing bacteria was carried out, and it was recognized

10) D. Watt and L. O. Krampitz, *Fed. Proc.* **6**, 301 (1947).
11) D. Bonner, *J. Biol. Chem.* **166**, 545 (1946).
12) E. A. Adelberg and E. L. Tatum, *Arch. Biochem.* **29**, 235 (1950).
13) E. J. Conway, "Microdiffusion Analysis and Volumetric Error", Crosby Lockweed & Son Ltd., London (1950).
14) E. Juni, *J. Biol. Chem.*, **195**, 715 (1952).

that in shake culture a rather large amount of L-valine is practically produced as the sole amino acid in the broth from carbohydrates and nitrogen sources by some species of bacteria.

2. Two strains of newly isolated L-valine accumulating bacteria were studied according to the system described in Bergey's manual, and named *Aerobacter cloacae* var sp. NISR-B-151 and *Aerobacter aerogenes* NISR-B-801, respectively.

3. L-Valine was isolated from the culture broth in the crystalline form and identified.

4. The high level of L-valine production is attributed to the newly isolated *Aerobacter* bacteria, yielding as much as 0.2 mole from one mole of glucose.

Acknowledgements The author wishes to express his thanks to Prof. Emeritus K. Sakaguchi, Prof. T. Asai, Prof. Y. Sumiki, and Assit. Prof. K. Aida, the Institute of Applied Microbiology, Univ. of Tokyo, for their kind guidance and valuable advice throughout this study. He is also very grateful to Director K. Mogi and Dr. M. Mogi for their encouragement and supportance throughout the course of this work. Inbebts also are due to the Institute of Applied Microbiology, Univ. of Tokyo, for elementary analysis and infrared spectra measurements.

Studies on L-Valine Fermentation*

Part II. Certain Factors Affecting the Production of L-Valine by *Aerobacter***

By Zenzirō Sugisaki

In the previous paper[1], the auther has reported that a high level of L-valine is produced directly from carbohydrates and nitrogen sources in culture media by newly isolated strains belonging to the genus *Aerobacter*.

From the standpoint of amino acid fermentation, L-valine production is practically considered as a one-product fermentation. But, optimal conditions for this fermentation have so far not been clearly defined for either surface or submerged fermentations. The purpose of the present report is to describe the optimal conditions with respect to factors favoring L-valine production in shake flask culture.

MATERIALS AND METHODS

Fermentation techniques *Culture Aerobacter aerogenes* NISR-B-801 was used mainly. This strain differs from *Aerobacter cloacae var. sp.* NISR-B-151, which is gifted with strong activity, in respect that the former results in a distinct yield of valine in response to the conditions employed. Hence, by using this strain, apparent advantage was found to emphasize optimal conditions for the production of valine. The descriptions concerning the latter are almost omitted from the present paper.

Nutrient agar was used as the stock slant medium.

Inoculum medium The medium used for inculum preparation contained glucose, 1 per cent; beef extract, 0.3 per cent; sodium chloride, 0.3 per cent; and had a pH of 6.6~6.8.

Inocula From fresh slant culture 3 to 5 loopfuls of this organism were transferred into 100 ml of liquid inoculum medium contained in 500 ml shake flask, and were incubated for approximately 24 hrs. at 30°C on a reciprocal shaker. Optimum of this seed culture was used as inocula.

Fermentation medium Throughout this work, glucose at level of 10 per cent and ammonium chloride were employed as carbohydrate and nitrogen sources, respectively. The concentration of ammonium chloride and mineral salts were made to vary as indicated in each figure. But the initial medium was the same as those used in screening test, i.e., 10% glucose, 1.2% NH_4Cl, 0.1% KH_2PO_4, and 0.05% $MgSO_4 \cdot 7H_2O$. The pH of all media was adjusted to about pH 6.4~6.6. The media were sterilized by autoclaving at 120°C for 5 min.

Fermentation An amount of about 2 to 3 ml of the vegetative incoulum, except as noted, was used for 100 ml of the appropriate fermentation medium in 500 ml shake flask, where a sufficient amount of calcium carbonate (3% w/v, sterilized separately) was also added to neutralize acidity produced during fermentation. Fermentations were carried out at 30±2°C on a reciprocal shaker (124 r.p.m.; Amplitude, 7 cm).

Analysis Analytical methods were exactly the same as those employed in the previous paper. Available oxygen was measured by *sulphide oxidation method* and indicated with Kd (oxygen absorption coefficient) according to the method of Yamada et al.[2]

RESULTS

According to the published data, it was recognized that the fermentative production of valine is greatly influenced by the various components of fermentation medium. For in-

*Reprinted by permission of the Inst. Appl. Microbiol., Univ. of Tokyo, from J. Gen. Appl. Microbiol. Vol. 6, No. 1.

**This work was presented at the meeting of the Agricultural Chemical Society of Japan held at Tokyo University, Feb., 28, 1959.

1) Z. Sugisaki, *J. Gen. Appl. Microbiol.*, **5**, 138 (1959).

2) K. Yamada, J. Takahashi and H. Okada, *J. Agr. Chem. Soc of Japan*, **27**, 704 (1953).

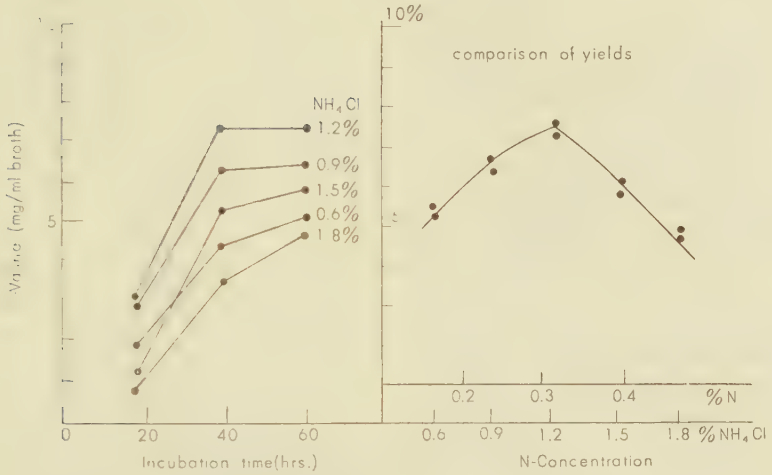


FIG. 1. Effect of Nitrogen concentration.
Basal med.: glucose 10%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, (pH 6.6); $CaCO_3$ 3%.
Seed. 3% (V/V)

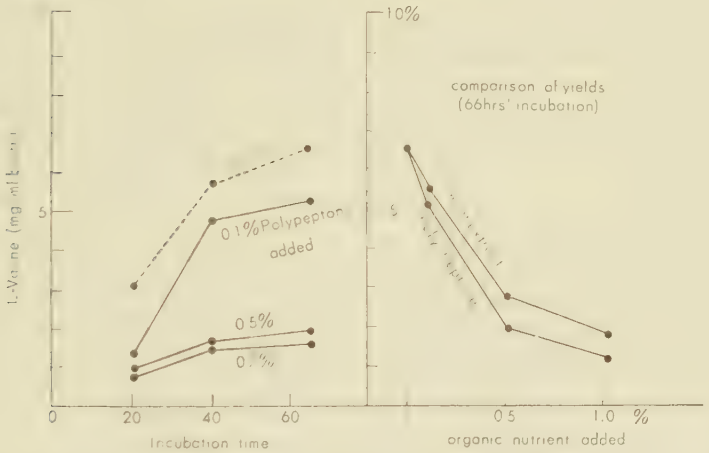


FIG. 2. Effect of addition of Organic-Nutrient.
Basal med.: glucose 10%, NH_4Cl 1.2%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCO_3$ 3%.
Seed: 2 ml in 100 ml broth.

stance, the yield of valine was considerably affected by the kind of nitrogen source; thence, in the present work, ammonium chloride was chosen.

Effect of Nitrogen concentrations To determine the optimum concentration of nitrogen, ammonium chloride was contained in each medium on several concentrations as noted in Fig. 1. It is evident that approximately 1.2 per cent of ammonium chloride, equivalent to 0.31 per cent of nitrogen, is optimum where glucose contained is at the level of 10 per cent (C/N=100 : 7.8). While glucose was at concentration of 2 per cent, 0.7 per cent of ammonium chloride was optimal (C/N=100 : 22.7).

Effect of supplied organic nutrients An attempt

was made to increase valine production by supplementing several organic nutrients such as beef extract, polypeptone, and yeast extract, etc. For example, the effect of polypeptone feed is shown in Fig. 2. The yield was highest in its absence or at its lowest level, and yet the period of fermentation was not shifted practically. In general, organic nutrients resulted in negative yields, except in some special cases of particular bacteria.

Effect of supplementing urea Udaka et al.[3] have reported that the addition of 1 to 2 per cent of urea (w/v) to the medium aids the formation of valine by *Paracolobactrum coliforme* (ureaseless). Actually, a little increase of valine production was caused by supplementing urea compared to that without it, with the use of our another strain, i.e., *A. cloacae* var sp. NISR-B-151 (urease, very weak). Presumably, this might be due to the pH control.

While, in case of *A. aerogenes* NISR-B-801, even a trace of valine was not obtained from the urea-supplied medium; owing to the strong urease activity of this strain, urea was rapidly decomposed and thus excess nitrogen rendered a toxic effect on bacteria.

Feeding method of nitrogen source It is essential for obtaining a good yield of valine to keep the bacterial growth adequately, which is affected by nitrogen concentration and aeration. So, the prescribed amount of nitrogen showed in Fig. 3 was distributively feeded at interval of 24-hrs. in each flask so that it amounted to 1.2 per cent of ammonium chloride in final. Then, bacterial growth and valine production were pretty influenced due to the initial level of nitrogen concentrations, especially at the early-stage of fermentation. However, it was difficult to recognize that a higher yield of valine was materialized at completion (see Fig. 3).

Effect of inorganic salts, bulk elements In the preceding sections, the effect of nitrogeneous constituents have been dealt with. In nature,

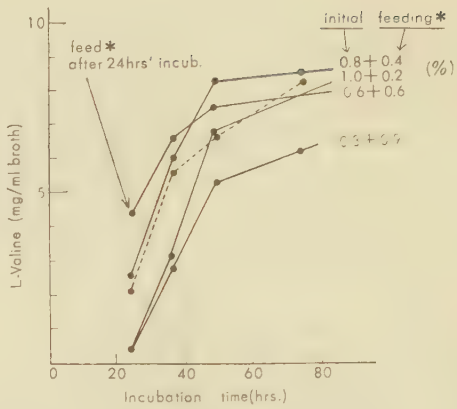


FIG. 3. Effect of N-feedings.

*Ammonium chloride was feeded at each concentration noted in figure after 24 hrs' incubation.
Basal med.: Glucose 10%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%; $CaCO_3$ 3%.
Seed: 3 ml in 100 ml broth.
●----●: control (1.2% NH_4Cl).

this strain grows well on artificial media, such as Ayer-Rupp-Johnson's medium, rather than it does on organic media like peptone water. Therefore, it is conceivable that the inorganic components of media may play important roles in the fermentation in question. In fact, inorganic phosphates and magnesium ions had particular effects on the production of valine.

(1) The optimal concentration of magnesium sulphate (Fig. 4): As shown in Fig. 4, it is evident that valine formation is severely affected by the amount of magnesium ion present. An amount of 0.04 per cent or less of magnesium sulphate has caused accelerative effect on valine formation, especially, at a later-stage of fermentation. In contrast to this, production was markedly depressed by an excess amount of magnesium salt.

(2) The optimal concentration of pottasium phosphate (Fig. 5): Phosphorus, present at phosphate ion, was required in a moderately high level and it gave a positive effect especially at an early-stage of fermentation. The optimal concentration of K-phosphate is about 0.2 per cent, and it is more effective where equal amounts of each mono- and di-pottasium phosphate are contained.

3) S. Udaka, K. Tomizawa and S. Kinoshita, *Presented at the meeting of Agricultural and Chemical Society of Japan*, at Univ. of Tokyo on Oct. 25, 1958.

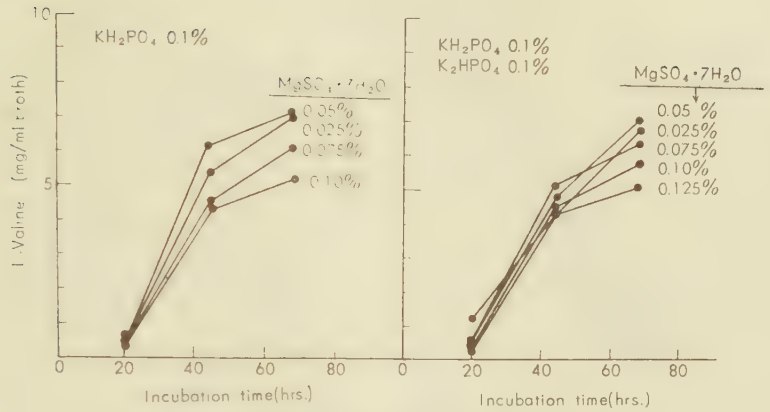


FIG. 4. Effect of Mg^{++} ion.
Basal med.: Glucose 10%, NH_4Cl 1.2%; $CaCO_3$ 3%.
Seed: 3 ml in 100 ml broth.

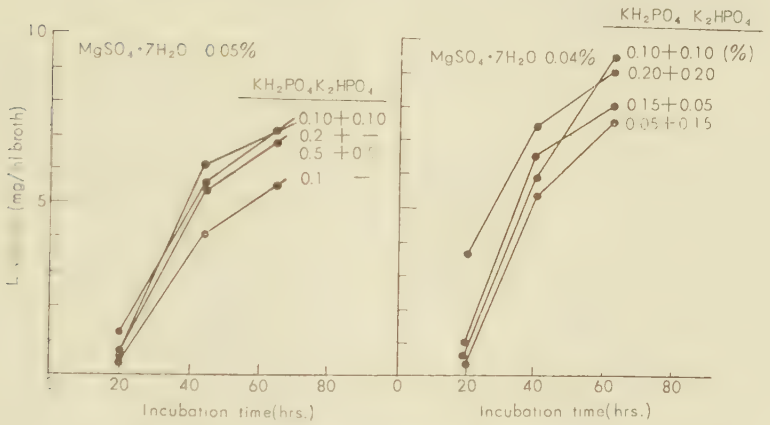


FIG. 5. Effect of K-Phosphates concentration.
Basal med.: Glucose 10%, NH_4Cl 1.2%; $CaCO_3$ 3%.
Seed: 3 ml in 100 ml broth.

In the optimal levels of concentration of both magnesium sulfate and pottasium phosphates, fermentations were successively conducted and resulted in increased consumption of sugar and a high yield of valine.

Effects of minerals, trace elements The influences of the addition of trace elements on the production of valine are illustrated in Fig. 6 and 7. No attempt was made to purify the salts (special-grade). The following salts were added at the level of 10 ppm, which is equivalent to about 2 ppm of each metalic ion: $FeSO_4 \cdot 7H_2O$, $CoSO_4 \cdot 7H_2O$, $NiSO_4 \cdot 6H_2O$, $MnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2Om$, $3CdSO_48H_2O$, and $Na_2MoO_4 \cdot 2H_2O$. Additional experiments were conducted with KCl and NaCl at a level of 0.05 M. Then, it was made clear that a low level of minerals has very pronounced and unexpected effects on the formation of valine.

(i) Single addition (Fig. 6). Each medium was supplemented with each mineral in single. It was observed that some trace elements (Ni^{++}, Mn^{++}, Mo^{+VI}, Cu^{++}, K^{+}) resulted in a *positive* effect, while others had a *negative* effect on the

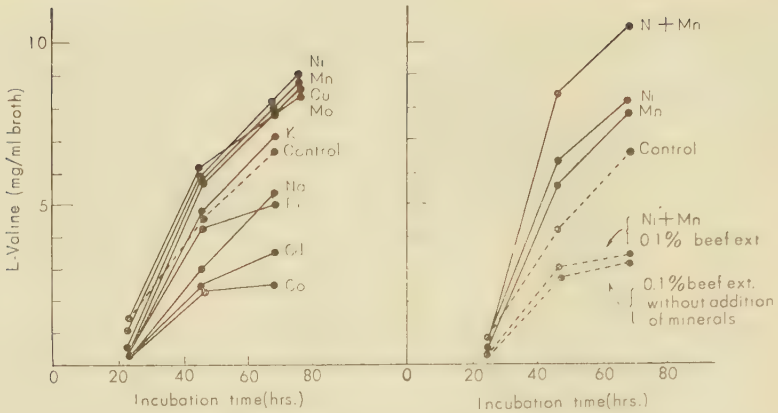


FIG. 6. Effects of Trace elements (Minerals).

Each mineral was supplied as sulfate salt, except molybdate, at concentration of 10 p.p.m. (equivalent to about 2 p.p.m. of metaric ion).
Basal med.: Glucose 10%, NH_4Cl 1.2%, KH_2PO_4 0.1%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.04%; CaCO 3%.
Seed: 3 ml in 100 ml broth.

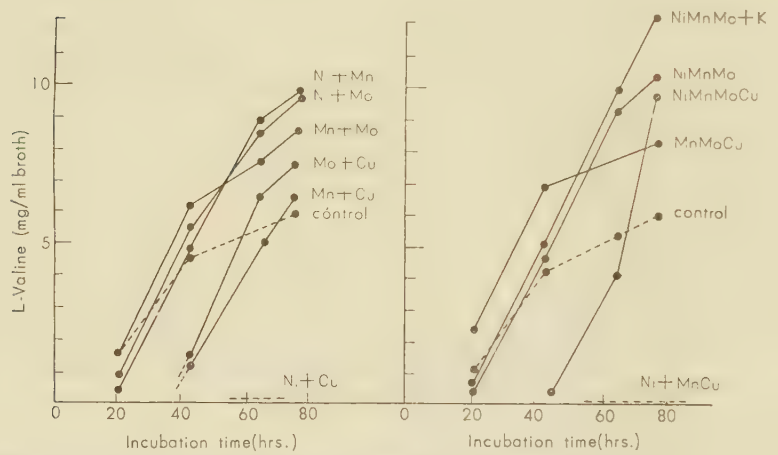


FIG. 7. Complex Effects of Minerals.

Basal med. and Seed: The same as those in the previous experiment, Fig. 6.

formation of valine as shown in Fig. 6. These results were confirmed in several subsequent fermentations.

(ii) Complex addition (Fig. 6, 7). The minerals which appeared to enhance valine production were supplied as a combination of several kinds of ions. It was distinctly recognized that in case of the addition of several minerals *more positive or negative* effects are involved than in the case of single mineral addition. It seems to be rather reasonable that every combination of Ni^{++}, Mn^{++}, Mo^{+VI} results in *positive* effects. However, it was an unexpected and a very interesting phenomenon that cupric ion resulted in *positive* effect in single addition, but had a remarkable *negative* effect in the case of combination with nickel ion as shown in Fig. 7. In the latter case, the bacterial

TABLE I. EFFECTS OF MINERAL IONS

Supplement	Effects: Positive effect	Effects: Negative effect
Single addition	Ni^{++}, Mn^{++}, Mo^{+VI}, Cu^{++}, K^{+} [mederate]*	Fe^{++}, Co^{++}, Cd^{++}, Na^{+} [moderate]*
Complex addition	Ni^{++}, Mn^{++}, Mo^{+VI}, K^{+} } various combinations [strong]*	Cu^{++} { $+Mn^{++}$, Mo^{+IV} [weak]*; $+Ni^{++}$ [strong]*

* Shows degree of *positive* or *negative* effects.

growth was practically depressed and valine formation was not recognized at all.

The effects of mineral ions are summarized in the accompanying table (Table 1).

On the maintenance of optimal pH A suitable neutralization of the acids formed during fermentation is necessary for good growth of bacteria and valine production. Excess calcium carbonate could be used in laboratory shake flask fermentations to provide continuous neutralization, which otherwise could not be readily accomplished. For valine fermentation, it is desired to maintain the pH-value as long as possible in the region of pH 6.2 to 5.4. This was practically attained by addition of calcium carbonate.

Combining the above results, one of the optimal media for L-valine fermentation by *A. aerogenes* NISR-B-801 will be as follows: 10% glucose, 1.2% NH_4Cl, 0.1% KH_2PO_4, 0.1% K_2HPO_4, 0.04% $MgSO_4 \cdot 7H_2O$, 10 ppm of each $NiSO_4 \cdot 6H_2O$, $MnSO_4 \cdot 7H_2O$, $Na_2MoO_4 \cdot 2H_2O$, and 0.35% KCl; $CaCO_3$ 3.5% (w/v) of the medium.

Chemical factors affecting valine production have mainly been dealt with in the preceding experiments. In the following paper, it will become evident that some other factors may also play a very prominent role as essential participants in this fermentation.

Optimal temperature Though the growth of bacteria has hardly been effected in the range of 27° to 35°C, the optimal temperature for valine fermentation is about 30°C. Thus, the temperature has been always maintained at 30°C±2°C in the course of this work.

Effect of inoculum size From the preceding results, it can be observed that the yields of valine vary with the amount of inocula (cf. Fig. 1~5). Therefore, quantities of seed culture from 1 to 10 per cent (v/v) were tested as inocula for each 100 ml of fermentation medium to determine the optimum inoculum size. Some results are given in Fig. 8. Amount of less than 3 ml of inocula caused rather slower fermentation, while more than 5 ml did excessive growth, and in both cases attendant yields were reduced. Thence, optimum inoculum size seemed to be 4~5 per cent (v/v) of the medium.

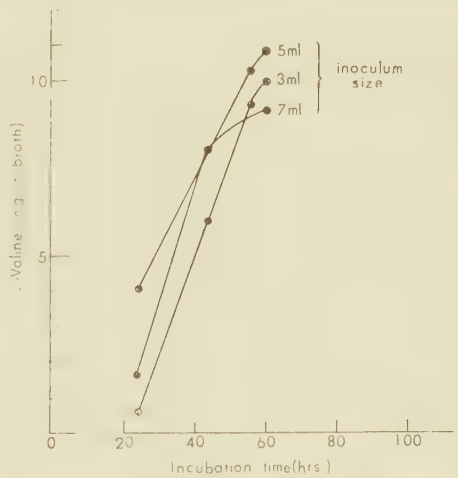


FIG. 8. Effect of Inoculum size.
Med.: Glucose 10%, NH_4Cl 1.2%, KH_2PO_4 0.1%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.04%, $NiSO_4 \cdot 6$ aq & $MnSO_4 \cdot 7$ aq 0.001% each; $CaCO_3$ 3.5% (sterilized separately).
Broth: 100 ml in 500 ml shake flask.

Effect of varying aeration Fermentations were run at several aeration levels in shake flasks with varied volume of medium, which were inoculated at 5 per cent level. The data of these fermentations are given in Fig. 9. Within

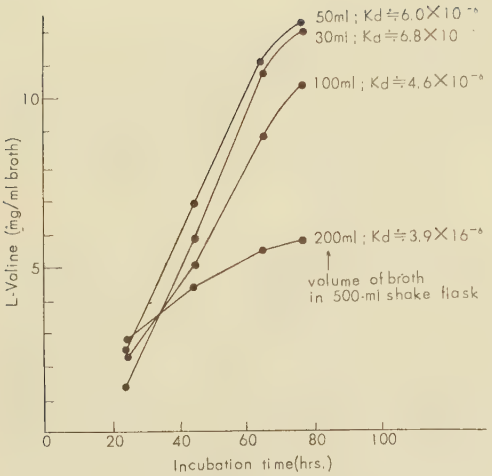


FIG. 9. Effect of Aeration.
Inoculum size: 5% (V/V of Broth.)
Kd: [g-mol of O_2/atm, min, cc of Broth].
Shaking: 124 r.p.m. 7 cm (30°C).

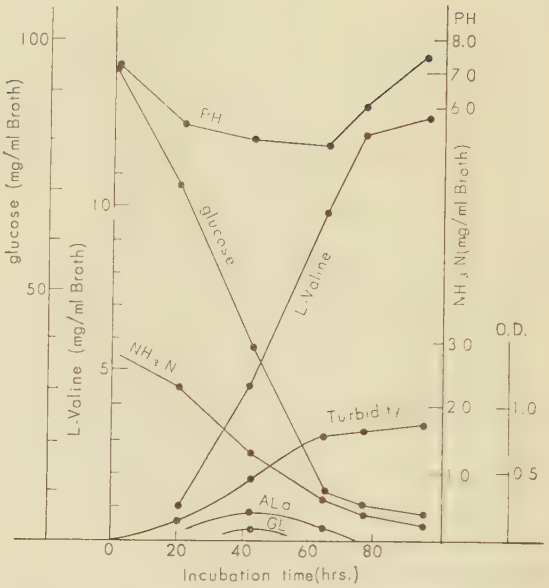


FIG. 10. Chemical Changes during Fermentation.
Strain: *Aerobacter aerogenes* NISR-B-801.
Med.: Glucose 9.4%, NH_4Cl 1.2%, KH_2PO_4 0.1%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.04%, $NiSO_4 \cdot 6$ aq, $MnSO_4 \cdot 7$ aq & $Na_2MoO_4 \cdot 2$ aq 0.001% each, KCl 0.4%; $CaCO_3$ 3.5% (sterilized separetely).
Broth: 50 ml in 500 ml Shake Flask.
Seed: 5% (V/V), Kd≒6×10^{-6} g-mol of O_2/atm. min, cc of broth.

the experimented range of aeration, a high level of aeration resulted in good growth of bacteria and a high yield of valine. It is evident from the data that the optimal aeration is computed at Kd, 6×10^{-6} [gram-mole of oxygen per atm. min. cc. of broth]. Similar effects were observed with *A. cloacae* var sp. NISR-B-151. Whilst, under anaerobic conditions, growth of bacteria and utilization of glucose were much slower than those conducted under aerobic conditions, and practically no valine formation was recognized but acetoin was extremely found.

Progressive fermentation by A. aerogenes NISR-B-801 An example of chemical changes during the fermentation is illustrated in Fig. 10, in which the data give the sequences of events occurring in one run employed under the optimal conditions fulfilled with several factors mentioned. Analysis performed successively during fermentation represent the rapid consumption of glucose and ammonia, variation of pH, and the formations of amino acids with adequate growth of bacteria under a sufficient aerobic condition. A small amount of glutamic acid and alanine were observed at the middle-stage of fermentation, but faded away in a later-stage. When glucose is nearly exhausted, pH tends to basic; then, valine production phase is drawing to a close.

Thus 1.26 gram of L-valine was produced from 9.0 gram of consumed glucose (initial glucose 9.4 gr. per 100 ml broth); that is, the yield of valine showed 14 per cent of consumed sugar or 13.4 per cent of total sugar content. If it is assumed that 1 mole of valine is formed from 1 mole of hexose sugar fermented, theoretical yield of valine should correspond to 65 per cent of glucose (w/w). Hence, from data, approximately 0.21 mole of valine was converted from 1 mole of glucose in this progressive fermentation.

DISCUSSION

As detected apparently in the previous report[1], L-valine is considered to be one of the normal products formed with species of *Aerobacter* and

other *Enteric* bacteria under the conditions studied.

The production of valine is probably the results of the interrelation of many factors. Formost among these are inherent factors; that is, the kind of bacteria. But strains of bacteria do not readily produce much valine and have to be *steered* to make appreciable quantities of this essential branched amino acid. Then, in the present report, *posteriori* factors affecting valine production—cultural conditions—were surveyed.

The external factors that promote valine formation appear to be:

(1) Suitable nitrogen source and C/N-ratio. Inorganic nitrogen is one of the main substrates just as other amino acid fermentations are. Such kind of nitrogen source and C/N-ratio are of crucial importance to valine production; the C/N-ratioes vary from about 100:23 to 100:8 with 2% and 10% sugar concentrations, respectively.

(2) Limited but adequate growth. Optimal inoculm size and maintenance of the cell with an adequate air supply are necessary conditions for suitable growth in this fermentation.

(3) Adequate aeration. It is apt to be considered that the fermentation may occur under relatively non-aerobic conditions, since known pathway[4,5] is hold of some reductive process. But such presumption would be completely exploded by the facts that the fermentation favored extremely aerobic condition, while, under anaerobic condition, acetion was the predominant product in stead of valine. Thence, aeration as well as nitrogen concentration is considered to be a leading factor bringing about this fermentation. In view of the known pathway of valine biosynthesis, this is the reason why aeration is necessary to produce the hydrogen-carrying co-enzyme (perhaps, TPNH) which is required for the formation of the reduction product, α,β-dihydroxy-isovaleric acid (DHV) from α-keto-β-hydroxy-isovaleric acid (KHV). This means that oxidative pattern, possibly tri-carboxylic acid cycle, must operate to a certain extent: but the final step in aerobic metabolism, complete oxidation of the dydrogen (TPNH) by excess amount of oxygen, should be restricted. The accumulated TPNH is then diverted to other reductions that give DHV. Aeration is also necessary for the formation of L-glutamic acid, NH_2-donnor of α-keto-isovaleric acid (KV).

(4) Presence of minerals. Not only bulk elements (phosphorus, magnesium and pottasium) but also trace elements (e.g., Ni, Mn, Mo, etc.) of mineral salts were required for progressive fermentation. Characteristically, a trace amount of Mn^{++} exerted a *positive* effect on the formation of valine, while an excess amount of Mg^{++} held its formation in check. This supports the opinion of Juni[8] who reported that Mg^{++} as well as Mn^{++} plays a role under pH 6 as cofactor in both condensation reaction of pyruvate to α-acetolactate (AL) and decarboxylation of it to acetyl-methyl-carbinol (AMC) in *Aerobacter aerogenes* (cf. Optimal pH of AL condensing enzyme is pH 8 in *E. coli*[10]).

(5) Maintenance of optimal pH. Reviewing the experimental data, it is desired to maintain the pH-value below 6.2 but not far below 5.4. With excess calcium carbonate, ammonium salts of strong acids, e.g., ammonium chloride or ammonium sulfate etc., serve to maintain the pH-value optimal.

A simultaneous increase in the ratio of valine to consumed sugar support the concept that certain initial products of the fermentation are utilized later in the formation of additional valine. Such metabolic products as pyruvic acid and probably active acetaldehyde may serve as intermediates for valine formation via AL and the following C_5-precursors, KHV→DHV→KV as described in the previous paper[1].

4) H. E. Umbarger and B. Brown, *J. Biol. Chem.*, **233**, 1156 (1958).
5) H. E. Umbarger, B. Brown and E. J. Eyring, *J. Am. Chem. Soc.*, **79**, 2980 (1957).
6) E. A. Adelberg and H. E. Umbarger, *J. Biol. Chem.*, **205**, 475 (1953).
7) D. Rudman and A. Meister, *ibid.*, **200**, 591 (1953).
8) E. Juni, *ibid*, **195**, 715 (1952).
9) H. E. Umbarger and N. Brown, *ibid*, **233**, 415 (1958).
10) H. E. Umbarger ann B. Brown, *ibid.*, **233**, 1156 (1958).

Moreover, in the course of fermentation, the disappearance of glutamic acid once formed suggests that transamination mechanism may be operation in the formation of valine as already reported[6,7]. Thus, both the present data and indicated facts support the postulated mechanism demonstrated with *Escherichia coli* mutant strains by Umbarger et al.[4,5] (cf. the accompanying scheme).

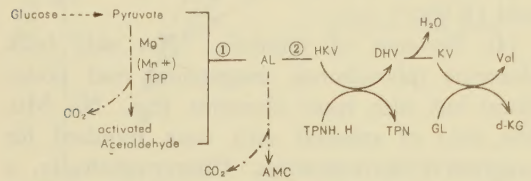


FIG. 11. The known postulated Mechanism of Valine biosynthesis and of Acetoin formation.

Abbreviations used here represent: TPP, Thiamine-pyrophosphate; GL, Glutamic acid; α-KG, α-Keto-glutaric acids; ①, Ketol Condensation; ②, Intramolecular Rearrangement; and other abbreviations are noted in the present text or previous report.

What is characteristic of this fermentation at completion is an almost constant proportion of formed valine, about 20 per cent conversion, to consumed sugar. And yet valine formation is depressed by the addition of organic nutrients. In a broad sence, this may fall under the category of "the so-called negative feed back control theory"[9]. Nevertheless, the high level of external accumulation of valine may be attributed to the formation of excess amount of it in bacterial cell and permeability of cell membrane —prominent features of this culture.

The mechanism of valine fermentation is currently under study in this laboratory.

SUMMARY

Factors affecting the fermentative production of L-valine were studied and optimal conditions were determined in the shake flask production by *Aerobacter aerogenes* NISR-B-801.

Optimal C/N-radio, 100:7.8 (at 10% glucose), and adequate aeration, Kd. about 6.0×10^{-6} [gram-mole of oxygen per atm. min. cc of broth], were found to be the most important factors to bring about L-Valine fermentaion. Chemical ingredients of media, especially trace elements (e.g., Ni, Mn, Mo) as well as bulk elements (phosphorus, Mg, K) have also remarkable influences on valine formation. The most efficient fermentation was performed with optimum inocula, 4~5 per cent (v/v) of media, under optimal pH 6.2~5.4.

The high yields of L-valine, about 20 per cent conversion of glucose, were obtained under the conditions studied.

Acknowledgements The author wishes to express his hearty thanks to Prof. Emeritus K. Sakaguchi, Univ. of Tokyo, and to Prof. T. Asai, Prof. Y. Sumiki, and Assist. Prof. K. Aida, the Institute of Applied Microbiology, Univ. of Tokyo, for their kind guidance and valuable suggestions throughout this work. The author is also very grateful to Dr. M. Mogi, Director of the Institute, and Dr. F. Yoshida, the Institute, for their encouragement and support kindly extended. Thanks are also due to Mr. K. Mizusawa for his technical assistance.

Report of the
NODA INSTITUTE
FOR SCIENTIFIC RESEARCH

昭和35年10月20日　印刷
昭和35年10月25日　発行

編集兼
発行者　野田産業科学研究所
千葉県野田市野田399

印刷者　(株)笠井出版印刷社
笠井朝義
東京都港区芝南佐久間町1の53